CANCER MODULATING PROPERTIES OF UNIQUE SOUTH AFRICAN HERBAL TEAS (ROOIBOS AND HONEYBUSH) IN SHORT TERM *IN VITRO* AND *IN VIVO* CARCINOGENESIS ASSAYS

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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ABSTRACT

This thesis provides the first scientific evidence on the cancer modulating properties of two unique South African herbal teas, rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*) utilizing *in vitro* as well as *in vivo* carcinogenesis assays by:

- Demonstrating the *in vitro* antimutagenic activity of aqueous extracts of the herbal teas against the metabolic activated mutagens, 2-acetylaminofluorene (2-AAF) and the mycotoxin, aflatoxin B_1 (AFB_1) as well as, to a certain extent, against the direct acting mutagen, hydrogen peroxide, utilizing the *Salmonella typhimurium* mutagenicity assay.

- Increasing the activity of hepatic drug metabolizing enzymes, glutathione S-transferase alpha and UPD-glucuronosyl transferase, and reduced the oxidative stress by stabilizing the level of reduced glutathione (GSH) resulting in an increased hepatic reduced to oxidized glutathione ratio (GSG:GSSG). No toxic effects were noticed in rats consuming the herbal teas for 10 weeks as their sole source of drinking fluid.

- Demonstrating the *ex vivo* modulation of 2-AAF- and AFB_1-induced mutagenesis by sub- cellular hepatic fractions of rats consuming the herbal teas in the *Salmonella* mutagenicity assay. Hepatic cytosolic fractions protected against mutagenesis of both mutagens, while the microsomal fractions exhibited a reduced capacity to metabolize AFB_1 to its active mutagenic metabolite.

- Providing evidence for the *in vivo* modulation of tumour promotion using the liver as well as the two-stage skin carcinogenesis animal models. The unprocessed herbal teas arrested proliferation of the placental form of glutathione-S-transferase ($GSTP^+$) altered cells as well as reduced the total number of enzyme altered foci in the liver of rats. Topical application of polyphenolic fractions of the various herbal teas prior to 12-O-tetra-decanoylphorbol-13-acetate (TPA) tumour promotion, reduced tumour formation in mouse skin initiated with 7,12-dimethyl-benz[a]anthracene (DMBA). The protective effect was illustrated by a decreased tumour incidence, a reduction in tumour volume as well as a delayed onset of

tumour development. The flavanol/proanthocyanidin content of the fractions could play a major role in the protection against skin tumour promotion.

- Proposing possible mechanisms whereby rooibos and honeybush herbal teas could exert their cancer modulating properties with respect to *in vitro* and *ex vivo* antimutagenicity, *in vivo* oxidative status and reduced tumour promotion.

- Providing evidence that the herbal teas mimic the cancer modulating properties of green and black teas although differences exist, presumably due to differences in the polyphenolic constituents.

- Suggesting that rooibos and honeybush herbal teas may play an important role as chemopreventive agents in the modulation of cancer.

UITTREKSEL

Hierdie tesis bevat die eerste ondersoek na die effek van waterige en polifenoliese ekstrakte van rooibos (*Aspalathus linearis*) en heuningbos (*Cyclopia intermedia*) op verskeie aspekte van kankerontwikkeling. Die twee kruietees is uniek aan Suid-Afrika en kan 'n belangrike rol speel in die voorkoming van kanker. Verskillende *in vitro* so wel as *in vivo* studies het die volgende getoon:

- Antimutageniese aktiwiteite teen die metabolies-geaktiveerde mutagene, 2-asetielaminofluoreen (2-AAF) en die mikotoksien, aflatoksien B_1 (AFB_1) in die *Salmonella typhimurium* mutagenisiteitstoets. 'n Beperkte mate van beskerming is ook verleen teen die oksidatiewe mutageen, waterstofperoksied, sonder metaboliese aktivering.

- Verhoogde aktiwiteite van die fase II ensieme, glutatioon S-tranferase alfa en UDP-glukuronidase, wat liggaamsvreemde verbindings metaboliseer. Die kruietees verlaag die oksidasietoestand soos weerspieel word deur 'n toename van gereduseerde glutatioon tot die geoksideerde vorm in die lewer van rotte wat 10 weke hierdie kruietees gedrink het. Die kruietees het geen toksiese uitwerking op die rotte gehad nie.

- Antimutageniese aktiwiteite van subsellulêre fraksies van die lewer teenoor 2-AAF en AFB_1 in die *Salmonella* toets. Die sitosolfraksie van die rotlewer bied beskerming teen die geïnduseerde mutagenese van beide mutagene, terwyl die mikrosomale fraksie ook die metaboliese aktivering van AFB_1 na die aktiewe mutageniese metaboliet verminder.

- *In vivo* modulering van kankerpromosie met behulp van bekende rotlewer en muisvel kankerontwikkelingsmodelle. In die lewermodel het die ongeprosesseerde kruietees beide die ontwikkeling en getal van $GSTP^+$ fokusse onderskeidelik vertraag en verminder. In die geval van die velkankermodel het aanwending van polifenoliese fraksies van die kruietees beskerming gebied teen die ontwikkeling van velkankers by muise. Die aantal en grootte van die tumors het afgeneem terwyl die verskyning daarvan ook vertraag is.

- Verskeie meganismes waardeur rooibos- en heuningbostee moontlik kanker kan moduleer word voorgestel. Verskille in die polifenoliese samestelling asook hul onderskeie konsentrasies kan 'n belangrike rol speel in die kankerveranderende effekte van die tees.

- Dat gereelde inname van rooibos- en/of heuningbostee moontlik 'n belangrike rol kan speel in die voorkoming van dieet- en omgewings-geïnduseerde kankers.

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My Heavenly Father for giving me the perseverance and courage to complete this study.

ABBREVIATIONS

2-AAF	2-acetylaminofluorene
ABTS	2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)
AFB_1	aflatoxin B_1
AhR	aryl hydrocarbon receptor
ALP	alkaline phosphatase
ALS	alkali labile site
ALT	alanine transaminase
AOM	azoxymethane
AST	aspartate transaminase
ATBC	alpha-tocopherol, beta-carotene cancer prevention study
BCC	basal cell carcinomas
BP	benzo[a]pyrene
BW(G)	body weight (gain)
C	(+)-catechin
CARET	beta-carotene and retinol efficacy trial
CHO	Chinese hamster ovary cells
CHP	cumolhydroperoxide
COX	cyclooxygenase
DEN	diethylnitrosamine
DMBA	7,2-dimethylbenz[a]anthracene
DNA	deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DSB	double strand break
E/A	ethanol/acetone
EBS	oestrogen binding site
EC	(-)-epicatechin
ECG	(-)-epicatechin-3-gallate
EGCG	(-)-epigallocatechin-3-gallate
EGC	(-)-epigallocatechin
ER	oestrogen receptor
FB_1	fumonisin B_1
FRAP	ferric reducing ability of plasma
G6PDH	glucose-6-phosphate dehydrogenase
GC	(+)-gallocatechin

Gpx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione (reduced)
GSSG	glutathione (oxidised)
GST(P)	glutathione-*S*-transferase (placental form)
H_2O_2	hydrogen peroxide
HUMN	Human Micronucleus Project
iNOS	inducible nitric oxide synthase
MBN	methylbenzylnitrosamine
MDA	malondialdehyde
MMS	methyl methanesulfonate
MMC	mitomycin C
MNRET	micronucleated reticulocytes
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NBMA	*N*-nitroso-benzylmethylamine
NCI	National Cancer Institute (USA)
NO	nitric oxide
$O_2^{\cdot}$	superoxide radical
ODC	ornithine decarboxylase
$^{\cdot}OH$	hydroxyl radical
ORAC	oxygen radical absorbance capacity
P450	cytochrome P450 dependent monooxygenases
PAH	polycyclic aromatic hydrocarbons
PAPS	3'-phosphoadenosine-5'-phosphosulfate
PB	phenobarbital
PH	partial hepatectomy
RLW	relative liver weight
ROS	reactive oxygen species
RNS	reactive nitrogen species
SAM	S-adenosylmethionine
SCC	squamous cell carcinomas
SOD	superoxide dismutase
SSB	single strand break
TBARS	thiobarbituric acid reacting substances

TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TEAC	trolox equivalent antioxidant capacity
TP	total polyphenol
TPA	12-O-tetra-decanoylphorbol-13-acetate
TRAP	total radical-trapping parameter
UPD-GT	uridine-5'-diphospho-α-*D*-glucuronic acid glucuronosyl transferase

TABLE OF CONTENTS

TABLE OF CONTENTS

CHAPTER 1

General Introduction

General Introduction

Cancer is currently the second leading cause of death in the western world. More than 5.5 million cancer cases are diagnosed each year in developing countries (1) of which approximately 50 000 are reported to the National Cancer Registry in South Africa. It is estimated that one in every four South African males and one in every five South African females will be affected by a cancer diagnosis in their lifetime (2). The five leading cancers in South Africa in declining incidence in males are prostate, lung, oesophagus, bladder and colorectal and in females are cervix, breast, colorectal oesophagus and lung (Fig. 1).

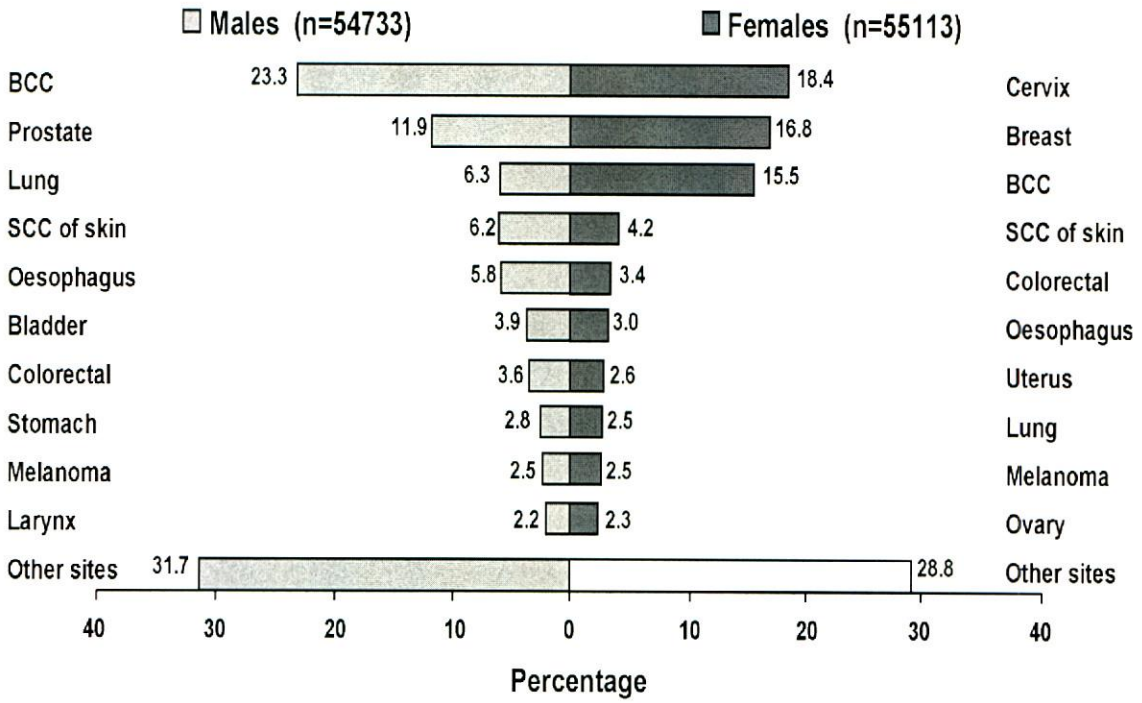

Fig. 1. Percentage distribution of the ten most common cancers in South Africa for period 1996 to 1997 (2).

Certain cancers are more common in certain geographic regions and population differences also exist. In a quantitative analysis of avoidable risks for cancer in the United States of America done by Doll and Peto (3), it was estimated that 35% of cancer deaths were related to diet, 30% to smoking and 35% to other causes, e.g. viruses, hormones, radiation, industrial carcinogens, etc. In the process of elucidating the causes of cancer, research also focussed on identifying compounds that might inhibit cancer development by interfering with one or more steps in carcinogenesis. Michael Sporn defined the term chemoprevention already in the mid 1970s (4) and research on chemoprevention started in all earnest in the early 1980s at the National

Cancer Institute, USA. Preclinical efficacy of more than 140 potential chemopreventive agents was evaluated during the 1980s and early 1990s and included agents such as antioxidants, anti-inflammatories, glutathione-S transferases and glutathione enhancers, phenolic compounds, etc. (5). Appropriate animal cancer models used to assess the efficacy of these compounds showed inhibition of chemical induced tumours in many organs and compounds having a high efficacy and low toxicity were selected for pre- and clinical trials (6). At present, phase 1, 2 and 3 clinical trials are directed at various cancers and include interventions with chemopreventive compounds or dietary substances (7). Future studies are also directed towards developing reliable biomarkers of exposure, determining bioavailability and reliable endpoints to be used in the clinical trials.

One of the approaches used in chemoprevention includes the intake of tea. Numerous reports indicate that the consumption of black and green teas (*Camellia sinensis*) has several health benefits e.g. reducing risk for heart disease and strokes as well as several lifestyle-related cancers (8-11). Apart from green and black tea, two indigenous herbal teas, rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*) have also been consumed in South Africa since the early 1700s. Anecdotal evidence of the health promoting properties of these herbal beverages has been accumulated. At present no scientific data are published on the biological activities of honeybush tea, while limited data are available on rooibos tea. Thus, the objective of this study was to substantiate some of the health claims and investigate the possible chemopreventive properties of these unique herbal beverages. Aspects of chemical carcinogenesis, chemoprevention and information about the chemical and biological properties of the herbal teas are reviewed in chapter 2. The *in vitro* antimutagenic activities and the *ex vivo* antimutagenic activities of rooibos and honeybush teas are discussed in chapters 3 and 5, respectively, while the *ex vivo* modulation of certain hepatic drug metabolizing enzymes and oxidative status are presented in chapter 4. The cancer modulating properties of rooibos and honeybush teas against cancer promotion in rat liver and mouse skin are provided in chapters 6 and 7, respectively. The key results of this study is summarised in chapter 8 which also includes some proposed future research directions.

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CHAPTER 2

Literature Review

Page

1. INTRODUCTION

Coronary heart disease and cancer are important disorders that cause alarming mortality and morbidity in humans (1). Both epidemiological and experimental carcinogenesis studies showed that more than 90% of cancers are associated with exposure to environmental mutagens and mitogens (2). Environmental factors implicated in cancer development in humans include cigarette smoke, physical carcinogens such as ultraviolet radiation, infectious agents such as pathogenic bacteria (*Helicobacter pylori*) and viruses (Human Hepatitis B and C virus) as well as dietary contaminants such as the mycotoxin, aflatoxin B_1 (3). The degree to which environmental factors effect tumour formation in humans will depend on the individual's susceptibility and defence mechanisms, e.g. DNA repair enzymes, antioxidant status and the level of detoxification enzymes (4-6). Some cancers (5-7% of total cancers), attributed to genetic factors rather than environmental factors, e.g. familial retinoblastoma, will not be discussed in the context of this review.

The discovery of the structure of DNA (7), contributed to our understanding of carcinogenesis at several levels. Cells divide and regenerate continuously and background or spontaneous mutations occur in approximately 1 in every 1,000,000 cells. Cells have the ability to repair damaged DNA, or to commit "suicide", referred to as apoptosis or programmed cell death, but when these protective mechanisms fail, tumours develop. Thus, damage of cellular DNA is essential to the process of cancer development and progression towards invasive cancer is characterised by accumulation of mutations and increased cell proliferation. The constitutive features of cancer include the ability of autonomous growth, to invade and metastasize. The process of carcinogenesis is usually prolonged, requiring one third-to-two thirds of the human lifespan to develop. Treatment of cancer is far more likely to succeed when diagnosed whilst still in the early stages.

Research efforts of the past four decades provide strong evidence that changes in dietary habits and lifestyles may reduce the risk for important diseases such as cancer. There is a growing interest in identifying natural occurring compounds capable of reducing the incidence of cancer, especially those caused by environmental factors. Tea is one of the most widely consumed beverages in the world today and its medicinal properties have been widely explored. Tea polyphenolic compounds have unique structures and activities for treatment of various

abnormalities and diseases in humans such as cancer, cardiovascular disease, dermatological problems, dental caries and even memory loss as shown by some epidemiological studies (8). Most of the information on the antimutagenic and anticarcinogenic activity of green and to a lesser extent black tea and their polyphenolic constituents is obtained from epidemiological and experimental studies. However, as mentioned, in South Africa, herbals teas derived from two endemic plants, *Aspalathus linearis* (rooibos tea) and *Cyclopia intermedia* (honeybush tea), are also consumed on a daily basis. These teas differ from green and black teas as they are naturally caffeine free, with a low tannin content and their phenolic composition differs not only from one another but also from that of green and black teas. The health promoting effects of these two endemic teas are mostly based on anecdotal evidence and sound scientific evidence is needed to substantiate these claims.

The present review mainly deals with the process of cancer development, polyphenolic compounds that may play a role in chemoprevention, some health benefits of green and black tea and the possible health promoting properties of the two unique South African herbal beverages, rooibos and honeybush teas.

2. Carcinogenesis

Percival Pott, an English surgeon is regarded as the father of carcinogenesis studies as he recognised that an environmental agent was responsible for the high incidence of scrotal cancer among chimney sweeps in London in the 1700's (9). Legislation then required bathing for removal of the soot and protective clothing for chimney sweeps resulted in the elimination of scrotal cancer as an occupational hazard. It was only in the early 1900's that scientists had developed an animal model to test the hypothesis that certain chemicals may cause tumours. Yamigawa and Ichikawa (10) demonstrated that skin carcinomas are induced by the chronic application of coal tar to the ears of rabbits. Cook and his co-workers (11) extracted the substance responsible for the carcinogenicity of coal tar on rabbit skin, called benzo[a]pyrene (BP). This substance has been studied extensively while numerous other occupational or environmental chemical carcinogens have been identified (Table 1).

Table 1 Chemical carcinogens identified by the National Toxicology Programme, United States of America

Chemical carcinogens	
Aflatoxins	Cyclophosphamide
Arsenic	
Asbestos	Diethylstilbestrol
Azathioprine	
Analgesic mixtures containing phenacetin	Eronite
Benzene	Methoxsalen with ultraviolet light
Benzidine	Mustard gas
Bis(chloromethyl)ether	
1,4-butanediol dimethyl-sulfonate	2-naphthylamine
Chlorambucil	Radon
2-chloroethyl-3-(4-methyl-cyclohexyl)-1-nitrosourea	
Chromium	Thorium dioxide
Conjugated estrogens	
	Vinyl chloride

Table modified from Perantoni *et al.* (9).

2.1 A multistage process

Cancer researchers today share views that cancer development is a multistage process with the accumulation of genetic changes a function of time (6,12,13). Carcinogenesis is often characterised by the following sequential stages: initiation, promotion, progression and malignant conversion (Fig. 1). Initiation is described as an irreversible consequence of the interaction of a carcinogen with tissue causing DNA damage, either through strand breaks or the formation of adducts (14-16). When a round of cell replication occurs before the DNA damage is repaired, the lesion is "fixed" and alterations can either be neutral or cause an error in the sequence, producing cells that are potential precursors of neoplastic growth. This step is often not recognisable as a pathological entity as the initiated cell may not be phenotypically different from the other parenchymal cells in the specific organ. Spontaneous initiation may also occur whereby the agent responsible for the initiating event is unknown, e.g., when cellular DNA is changed at one or more sites in the genome as a result of incorrect action of DNA polymerases during cell division or during DNA repair. These changes represent a mutational event that may be hereditary. In different model systems, initiated cells exhibit certain characteristics, when compared with the surrounding non-initiated cells including increased proliferative capabilities, resistance to apoptotic stimuli and inducers of cell cytotoxicity resulting in an increased lifespan (17). Initiation alone does not result in tumour formation as the development into a clinically detectable neoplasm requires several additional events.

The next stage, referred to as cancer promotion, is defined as the step where specific agents known as promoters selectively enhance the development of neoplasms from a background of initiated cells (18). Promotion is suggested to be reversible as withdrawal of the agent usually results in the disappearance of the expanding clones of initiated cells, e.g. the regression of polyps in the colon. Thus, the promotion process facilitates the expression of the initiated phenotype and is not considered to be genotoxic (19). Promoters are generally not mutagenic and may be agents of exogenous origin, such as therapeutic drugs and plant products, or endogenous origin, including hormones or chronic inflammation (18). These agents do not directly interact with the host cellular DNA but influences the expression of genetic information encoded in the cellular DNA, referred to as epigenetic mechanisms. Certain promoters produce their effect by interaction with receptors in

the cell membrane, cytoplasm or nucleus and others are mitogenic by stimulating DNA synthesis and enhancing cell proliferation (20). Promoters also appear to have relatively high tissue specificity, e.g. phenobarbital functions as a promoter for rodent liver neoplasia but not in the urinary bladder (21). Similarly, the phorbol ester, 12-*O*-tetra-decanoylphorbol-13-acetate (TPA), is a potent skin and forestomach cancer promoter but has no appreciable activity in the liver (18). Some promoters cause inflammation and this is particular relevant in skin initiation-promotion studies using the phorbol esters. Tumour promotion may be modulated by several factors such as age, sex, diet and hormone balance (18,22).

A second genetic event and/or series of events proposed to increase the probability of a cell to become malignant; occur during the third stage referred to as progression. The DNA changes involved could arise from additional exposure to carcinogens, spontaneous mutations or genomic instabilities resulting in an irreversible change in the phenotype of a cell (6,12,23).

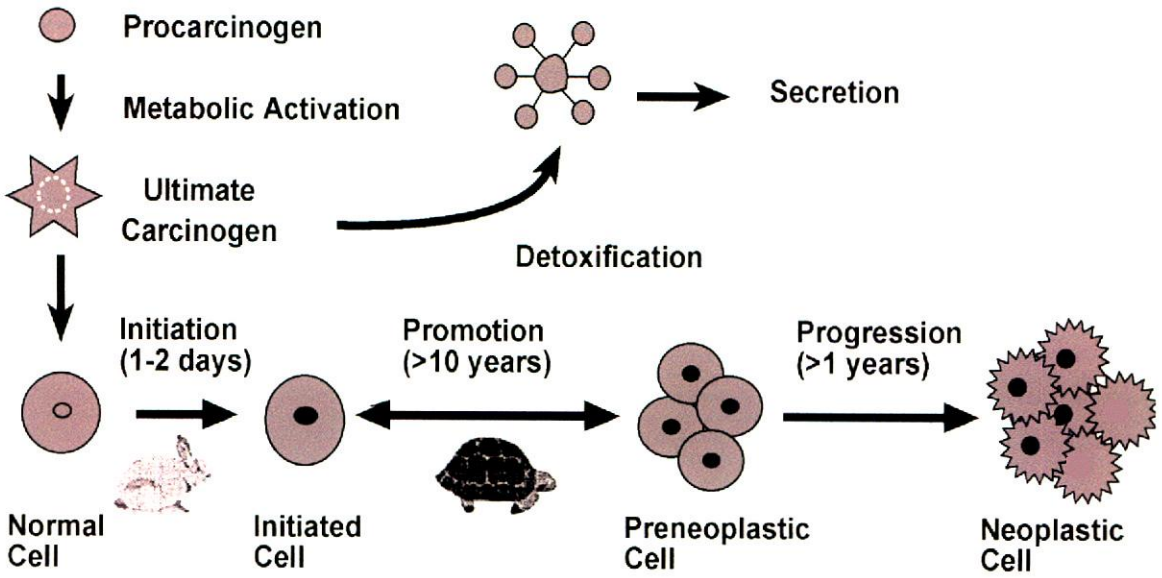


Fig. 1. Sequential stages of carcinogenesis (24).

Progression is used to signify the stage whereby a benign proliferation becomes malignant or whereby a neoplasm develops from a low grade of malignancy to a higher grade (9,17). During progression, neoplasms show increased invasiveness, develop the ability to metastasize and alter biochemical, metabolic and morphological characteristics. Tumour cell heterogeneity is an important characteristic of tumour progression (18). Associated with progression is the

development of an increased degree of karyotypic instability as well as chromosomal rearrangement. Malignant conversion may be considered the final step in progression. Distinction between tumour promotion and tumour progression is not clear when performing a routine histopathological evaluation of neoplasms. In both situations though, the critical event is accelerated growth (19). Emerging technologies offer promise to help distinguish various stages of progression.

A complete carcinogen is an agent capable of providing initiation through single or multiple exposures, promoting a growth advantage to selective altered cells resulting in lesions generated during progression that have the potential to undergo malignant transformation.

2.2 Short-term *in vitro* mutagenesis and *in vivo* carcinogenesis models

2.2.1 *In vitro* mutagenesis testing

Prior to performing carcinogenesis assays in animal models numerous *in vitro* test systems exist to evaluate the carcinogenic potency of various synthetic chemicals and/or natural compounds. The predictive power of *in vitro* mutagenicity assays to identify potential carcinogens is then often evaluated through comparison with carcinogenic assays in rodents. Short-term assays include bacterial tests, e.g. *Salmonella typhimurium* mutagenicity or Ames assay; mammalian DNA mutagenesis and repair tests, e.g. COMET assay; and chromosome integrity tests, e.g. micronucleus and sister chromatid exchange assays. Table 2 lists some of the most widely used *in vitro* assays. Some chemicals exhibit their carcinogenic effects by mechanisms that do not involve DNA damage and are referred to as non-genotoxic carcinogens, e.g. estrogens, metals (12,25). There are a few *in vitro* methods to detect non-genotoxic carcinogens but none of these has been validated for regulatory usage. Chemicals that prove to be non-genotoxic *in vitro* and *in vivo* may still be subjected to a rodent bioassay, especially if a high level of human exposure is anticipated.

Table 2 Principal short term assays in assessing genotoxic effects

	Type of assay	Evaluating endpoint
Main assays	• *Salmonella*/AMES assay	Gene mutations
	• Micronucleus assay in rodent bone marrow	Chromosome damage *in vivo*
Other assays	• *E. coli* WP2 tryptophan reversion assay	Gene mutations
	• TK or HPRT forward mutation assay in cultured mammalian cells	
	• *Drosophila* sex-linked recessive lethal assay	
	• Chromosome aberrations and micronuclei assays	Cytogenesis in cultured hamster or human cells
	• Aneuploidy assays	
	• Mitotic recombination in yeast and *Drosophila*	Other indicators of genetic damage
	• Unscheduled DNA synthesis in cultured hepatocytes and rodents	
	• Mouse visible/electrophoretic specific-locus tests	Mammalian germ cell assays
	• Skeletal or cataract mutation assays	
	• Cytogenetic analysis and heritable translocation assays	
	• DNA damage and repair in rodent germ cells	
	• Dominant lethal assay	

Abbreviations: *E .coli* = *Escherichia coli*; TK = thymidine kinase; HPRT = hypoxanthine-guanine phosphoribosyltransferase. Table modified from Hoffmann *et al.* (26).

The percentage of chemical agents positive in the *Salmonella* mutagenicity assay that have been shown to be carcinogenic is approximately 83 to 89% (27,28). Therefore in addition to bacterial assays, the impact of mutagenesis in human health should encompass additional tests including gene mutations, chromosome aberrations and aneuploidy, in other test systems. A few of these short term *in vitro* and *in vivo* assays will briefly be discussed.

2.2.1.1 *Salmonella* mutagenicity assay

The study of mutations in bacteria has played a very important role in the science of genetics. The *Salmonella* mutagenicity assay developed by Bruce Ames (29) is the best-studied short-term predictive test for carcinogenicity and has been validated in studies by the National Cancer Centre Research Institute of Tokyo as well as the

International Agency for Research on Cancer (30,31). A range of *Salmonella typhimurium* strains with well-defined mutations in known genes became available (29). Each of the strains carries one of a number of mutations in the operon coding for histidine biosynthesis. The mutations can be reverted either by base-pair substitutions or frameshifts referred to as point mutations. For these commonly used strains the DNA sequence at the site of the original mutation in the histidine biosynthesis gene has been determined. Apart from these mutations in the histidine operon, the tester strains also contain other mutations that increase their ability to detect possible mutagens, e.g. the *rfa* mutation to increase membrane permeability to larger molecules and the *uvrB* mutation coding for the DNA excision repair system. A mutant strain is treated with a compound to determine whether a second mutation will directly reverse or suppress the original mutation in the histidine gene.

The standard tester strains normally used are TA 97, TA 98, TA 100 and TA 102. TA 97 and TA 98 are used for detecting various frameshifts, TA 100 for detecting base pair substitutions (adenine-thymine base pairs) and TA 102 containing the ochre mutation to efficiently identify other oxidative mutagens not detected or poorly detected by the other strains, e.g. various hydroperoxides, UV light, X rays and cross linking agents (27). These strains also contain the R-factor plasmid, pKM101 making them more susceptible to detect certain mutagens. Additional tester strains are available that do not contain pKM101, e.g. TA 1535 and TA 1538 that have a lower spontaneous mutation frequency than TA 100 but detect mutagens that do not revert TA 100. An enzyme component representing mammalian drug metabolism is also included.

Methods as well as revised methods for detecting mutagens and carcinogens using the *Salmonella* mutagenicity assay are described in detail in the literature (27,32).

2.2.1.2 Micronucleus assay

The use of micronucleus formation as a measure of chromosome damage in peripheral blood lymphocytes was first proposed by Countryman and Heddle (33) and has subsequently led to the development of the cytokinesis-block micronucleus method. The bone marrow of rodents is routinely used in this test since polychromatic (immature) erythrocytes are produced in this tissue. The measurement of

micronucleated immature erythrocytes in peripheral blood is equally acceptable in species in which the inability of the spleen to remove micronucleated erythrocytes has been demonstrated. The micronuclei are small membrane bound DNA fragments and originate from acentric fragments (chromosome fragments lacking a centromere) or whole chromosomes that are unable to migrate with the rest of the chromosomes during the anaphase of cell division. Micronuclei can be distinguished by a number of criteria, but the frequency of polychromatic erythrocytes is the principal endpoint. Cells are exposed to a test substance both with and without metabolic activation. Following exposure, cytochalain B is added to block cytokinesis and the cells are grown for a sufficient time to allow chromosomal damage and the formation of micronuclei in bi- or multinucleated interphase cells. The harvested and stained cells are analysed microscopically for the presence of micronuclei.

This assay can be applied to both *in vitro* as well as *in vivo* experimental conditions. The mammalian *in vivo* micronucleus assay is especially relevant to assess mutagenic hazards, as it allows consideration of factors of *in vivo* metabolism, pharmacokinetics and DNA-repair processes (34,35). This short term assay was also used in the Human MicroNucleus (HUMN) project to establish a database of "normal" baseline frequencies of DNA damage in human populations (36).

2.2.1.3 COMET assay

The COMET assay, also known as the single cell gel electrophoresis assay (SCGE), was developed in 1984 for identifying agents exhibiting genotoxic activity by detecting DNA damage at the level of the single cell (37). The original method was performed under neutral conditions, but subsequently a COMET assay performed under alkaline (pH >13) conditions was developed (38). In the COMET assay the cells are embedded in a thin agarose gel on a microscope slide and lysed to remove cellular proteins. The DNA is then allowed to unwind under neutral or alkaline conditions where after it is electrophoresed and stained with a fluorescent dye. The electric current causes migration of the broken DNA fragments or relaxed chromatin away from the nucleus, thus the COMET assay essentially measures the sizes of the various DNA fragments within that cell. The types of DNA damage detected by the COMET assay include double strand breaks at neutral conditions, single strand breaks at pH 12.1 and alkali labile sites at pH >13. Breaks can also be introduced at

specific sites with specific glycosylases and endonucleases. The COMET assay is also applied as a simple DNA repair assay suitable for use in biomonitoring. In an *in vitro* cellular assay cells are embedded in agarose, treated with a DNA-damaging agent and immersed in culture medium at 37°C to initiate repair in the gel (39). The applications of this technique include genetic toxicology, radiobiology, clinical investigations as well as environmental biomonitoring (40).

It is important to note that the use of the COMET assay for human biomonitoring is still controversial as single strand breaks are not the major form of damage induced by most genotoxins, and if they are, these breaks are rapidly repaired. However, the assay is regarded as a good addition to the existing test systems used in human biomonitoring studies.

2.2.1.4 Sister chromatid exchange assay

The sister chromatid exchange (SCE) assay provides a highly sensitive means of evaluating cytogenetic damage caused by chemical or physical mutagenic agents. SCE means a reciprocal interchange of the two chromatid arms within a single chromosome. The exchange is visualised during the metaphase of the cell cycle by differential staining and presumably requires the enzymatic incision, translocation and ligation of at least two DNA helices (41). Evidence of such exchanges appears as a banding effect. An increase in the number of exchanges observed in the test cells is an indication of a genotoxic effect. This assay can also be applied *in vivo*, e.g. in peripheral blood lymphocytes cell cultures from study animals (42).

2.2.2 *In vivo* carcinogenesis assays

The use of experimental animals as models for human disease has been indispensable in understanding the causes, biology and prevention of cancer and plays a crucial role in the preclinical testing of new anti-cancer drugs and treatments (43). Rodents are the most commonly used animals for modelling human tumours as they have several advantages in medical research. Their physiology and genetics are well characterised and understood, they are relatively easy and cheap to maintain, are mammals and readily develop cancer in response to chemical carcinogens over a short time period (44). Animal models also have their disadvantages when they are used to study human cancers. Extensive inbreeding has made rodent strains

genetically similar, whereas humans are genetically diverse and might respond differently when subjected to the same circumstances. Animals and humans have important metabolic, physiological and hormonal differences which will affect the type of carcinogens metabolised, the resultant metabolites as well as the subsequent repair of DNA damage (45). The practical limitations on the design of the animal experiments can make the findings difficult to apply to humans e.g. animals are usually exposed to higher levels of carcinogens than levels encountered by humans. The accepted design thus for carcinogenicity testing is to expose a statistically acceptable number of rats or mice to the highest dose they will tolerate without reducing their lifespan for reasons other than tumour development (46). A number of specific "short term" *in vivo* rodent carcinogenesis models have been developed to assess the carcinogenic potential of chemicals. A few of these models relevant to the present study will be briefly discussed.

2.2.2.1 Hepatocarcinogenesis in rats

A variety of carcinogenesis treatment protocols have been proposed in rat liver and is especially useful to identify potential liver tumour initiators and promoters (21,47-53). The first experimental models were developed by Peraino *et al.* (21) where the rat liver was initiated with 2-acetylaminofluorene (2-AAF) and exposed to phenobarbital (PB) to develop preneoplastic and neoplastic lesions. Subsequently, Pitot *et al.* (47) combined the partial hepatectomy and diethylnitrosamine (PH/DEN) method of Scherer and Emmelot (48) with the PB-promoting method of Peraino *et al.* (21) to clearly distinguish the stages of initiation and promotion. Foci of enzyme altered hepatocytes and/or hepatic nodules were used as the endpoint. The resistant hepatocyte model was developed by Farber *et al.* (49) and is based on the hypothesis that carcinogens induce "resistant" hepatocytes during initiation which are stimulated to develop into foci and nodules by brief exposure to carcinogens such as 2-AAF coupled with a mitogenic stimulus. This assay consists of 3 components, an initiator (e.g. DEN), a selective growth inhibitor, 2-AAF and a growth stimulus (e.g. PH). Male Fischer 344 rats are given a single dose of 200 mg/kg body weight DEN (intraperitoneally), after a 2 week recovery period from the initial cell damage, rats were fed a basal diet containing 0.02% 2-AAF for two weeks including a 67% PH after the first week (49). Within 7-10 days foci and/or nodules occurred randomly throughout the liver. The number of foci and/or nodules of enzyme altered (gamma-

glutamyl transpeptidase -GGT; glutathione-S-transferase placental form -GSTP) hepatocytes visualized histochemically, varied with the dose of DEN administered. Other models, including the choline-deficient model developed by Sells *et al.* (51) and the orotic acid model developed by Sarma *et al.* (53), have also been described in the literature. Subsequently, numerous chemicals have been identified and used as cancer initiators and/or promoters in these models, e.g. aflatoxin B_1 (AFB_1), and fumonisin B_1 [FB_1] (54-59).

2.2.2.2 Two-stage mouse skin carcinogenesis model

The initiation-promotion developed protocol in mouse skin carcinogenesis has been widely used for studies on mechanisms involved in chemical carcinogenesis and to evaluate the carcinogenic or anticarcinogenic properties of compounds (60,61). Initiation is accomplished by a single topical application of a sub carcinogenic dose of a carcinogen e.g. 7,2-dimethylbenz[a]-anthracene (DMBA) inducing a permanent genetic alteration in some epidermal cells (62). Repeated application of the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA) to mouse skin, leads to sustained proliferation resulting in hyperplasia in non-initiated skin and papilloma development in initiated skin. A fraction of these papillomas progresses to carcinomas through the acquisition of additional genetic changes (63). The progression stage is generally a spontaneous process (62,64).

2.2.2.3 Other models

Several other organ specific models have been developed in rats and mice for studying cancer development. Azoxymethane (AOM) is highly effective in causing colon and rectal cancers in rats (65). Male Fischer rats are injected subcutaneously with AOM and depending on the experimental design the aberrant crypt foci or other types of cancerous lesions are manifested in the colon (66-68). AOM-induced tumours were shown to share many histopathological characteristics with human tumours (69). Animal models have also been developed to study oesophageal carcinoma. Among the most potent inducers of oesophageal tumourigenesis in the rat is *N*-nitroso-benzylmethylamine (NBMA) and which is injected subcutaneously 3 times a week for 5 weeks (70-72). Induction of papillomas is monitored after 20-40 weeks.

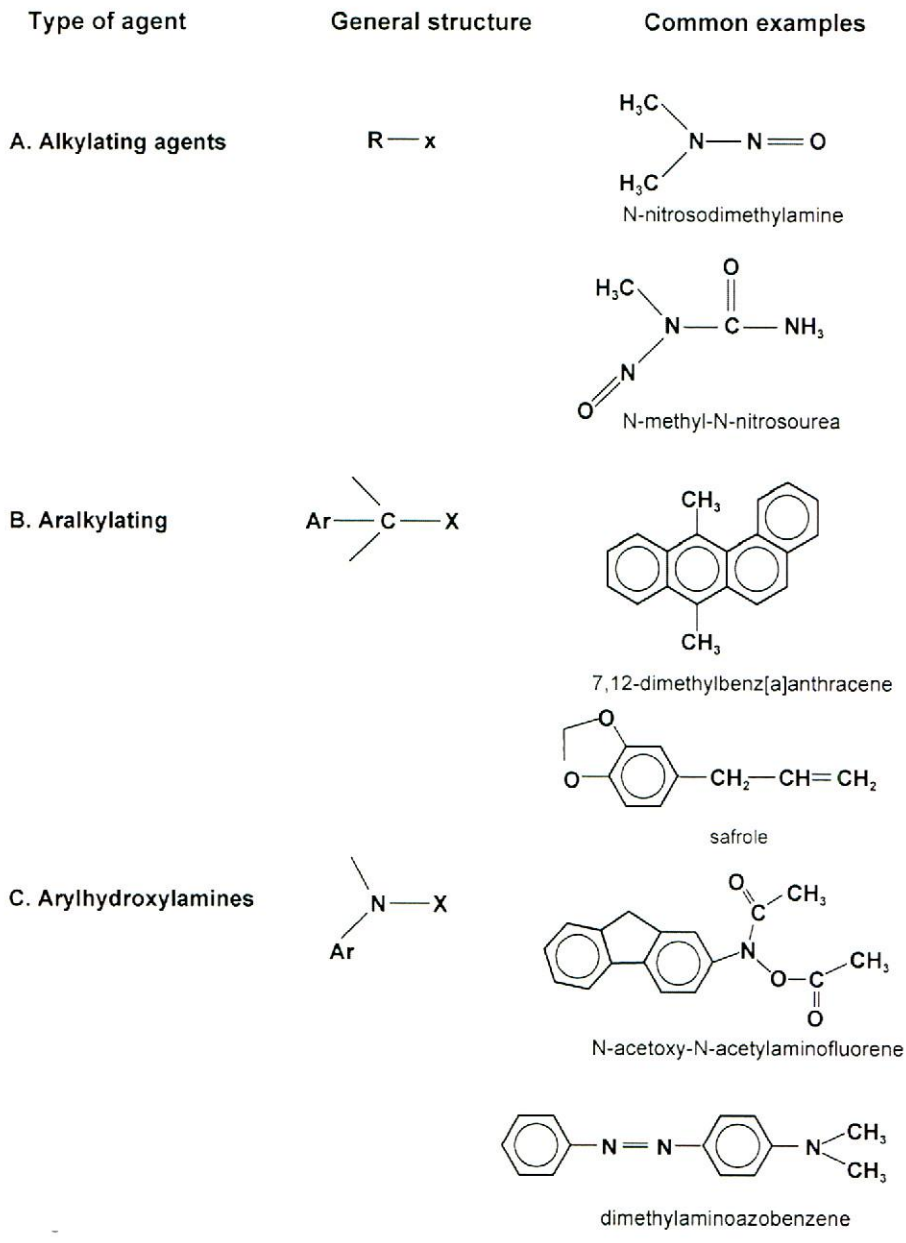


Fig. 2. The three major groups of chemical carcinogens (9).

The reactive metabolite is suggested to be a diol-epoxide, generated by two metabolic cycles involving P450. The epoxide intermediates are stable to allow movement to the cell nucleus where they react with DNA (79).

2.3.1.3 Arylhydroxylamines

The carcinogenic activity of the aromatic amines has been suggested from epidemiological studies of workers occupationally exposed to aniline dyes and the subsequent development of bladder cancer (80). One of the aromatic amines, 2-

acetylaminofluorene (2-AAF) was identified and listed as a known carcinogen in 1987 under the Safe Drinking Water and Toxic Enforcement Act of 1986 -Proposition 65 (81).

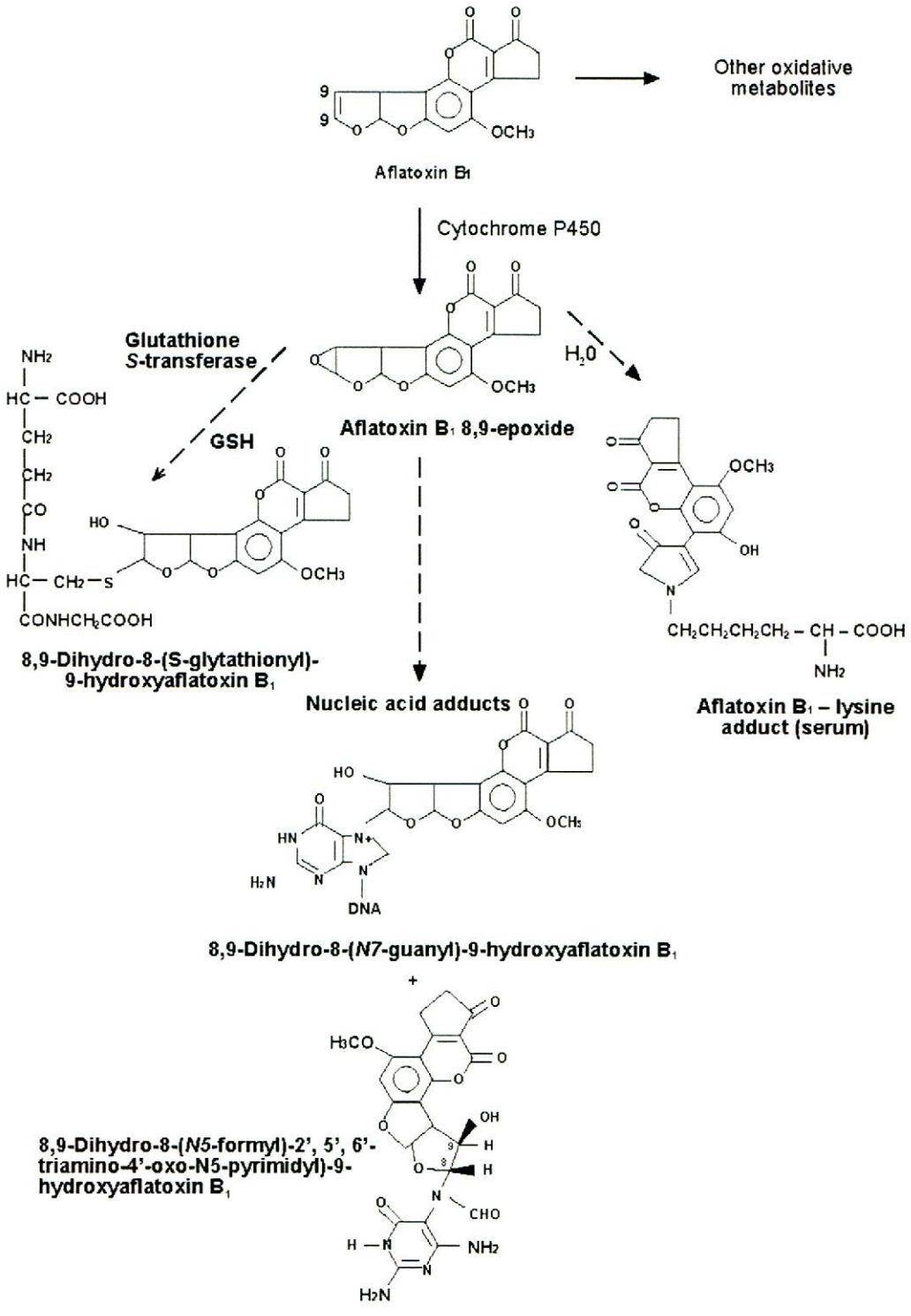


Fig. 3. The metabolism of Aflatoxin B_1 (75).

2-AAF induces tumours in the liver of male rats, mammary gland in female rats and ear duct gland and small intestine in both male and female rats (82).

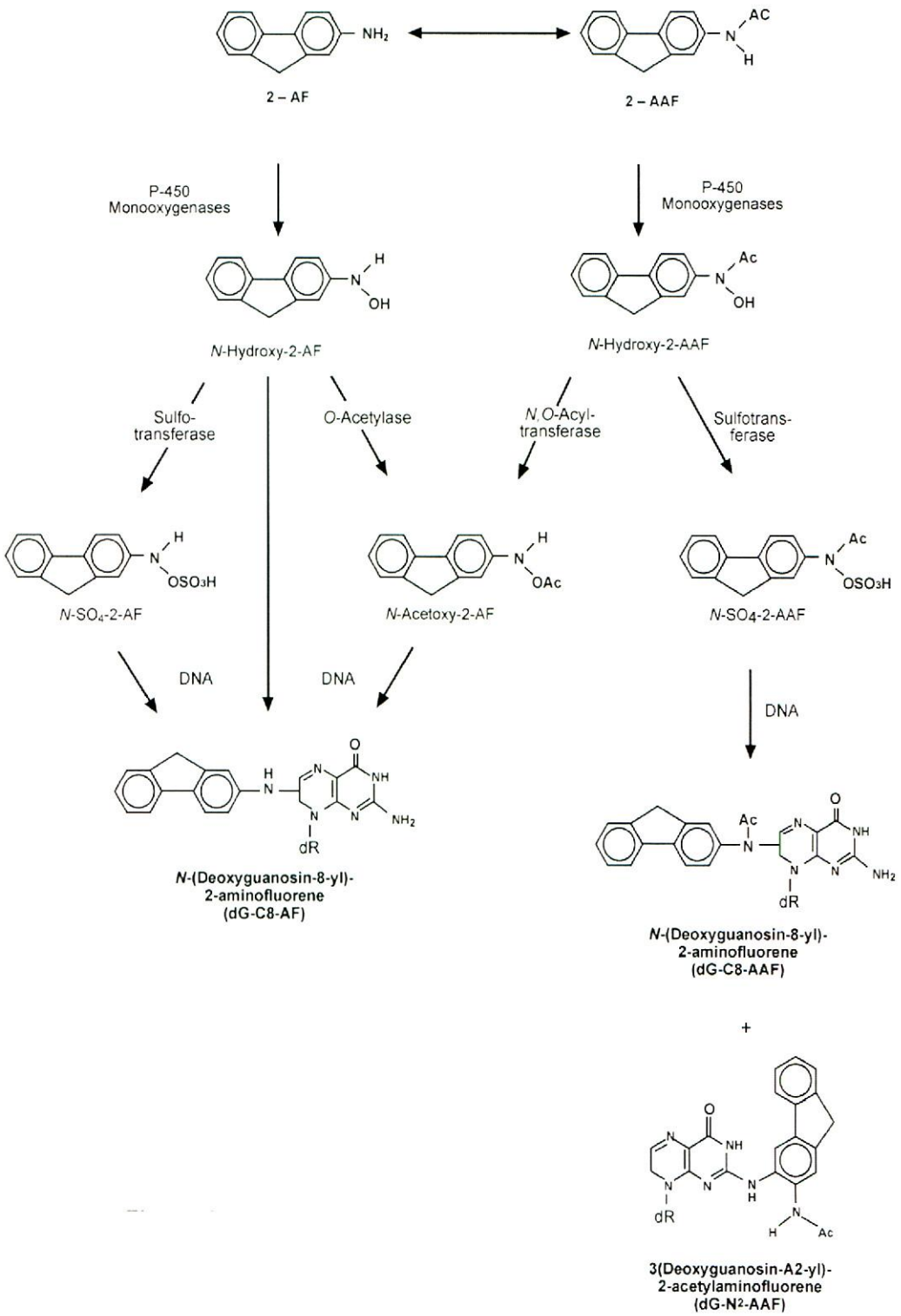


Fig. 4. The metabolism of 2-acetylaminofluorene in the rat liver by cytochrome P450 1A2 (83).

It is N-hydroxylated in the rat liver by CYP1A2 to N-hydroxy-2-acetylaminofluorene (Fig. 4) which can either be esterified to yield the sulphuric acid ester (N-SO_4-2-AAF) catalysed by the sulfotransferase system or be subjected to an intramolecular transfer of the acetyl group via acetyltransferase to N-acetoxy-2-AAF (83). All these metabolites may form adducts with DNA resulting in damage (84).

2.4 Metabolism of xenobiotic compounds

After a xenobiotic, e.g. chemical carcinogen, has been absorbed into a biological system it may undergo a biotransformation. The products of metabolism are usually more water soluble to facilitate their excretion via the kidneys or bile. If the compound is highly lipophilic, e.g. polychlorinated biphenyls and thus not suited for excretion, it will remain in the biological system for long periods of time (85,86). Metabolism of xenobiotics is generally divided into two phases: phase I and phase II (Fig. 5), with phase I altering the compound by adding a functional group which can be conjugated with endogenous polar constituents during phase II (85,87). Some xenobiotics already possess a functional group, e.g. hydroxyl groups and therefore simply undergo a phase II reaction. The products of phase II may be further metabolised involving both phase I and phase II enzymes.

The xenobiotic/foreign compound enters the body via the gastrointestinal tract and goes directly to the liver, the major organ involved in carcinogen metabolism via the portal blood supply. The metabolising enzymes are generally localised in particular subcellular fractions, e.g. in the endoplasmatic reticulum, in the cytosol and in other organelles such as mitochondria. The metabolic reactions produce unstable positively charged intermediates (electrophiles) which are chemically reactive. The reactivity of the metabolic intermediates may determine the toxicity of the parent compound.

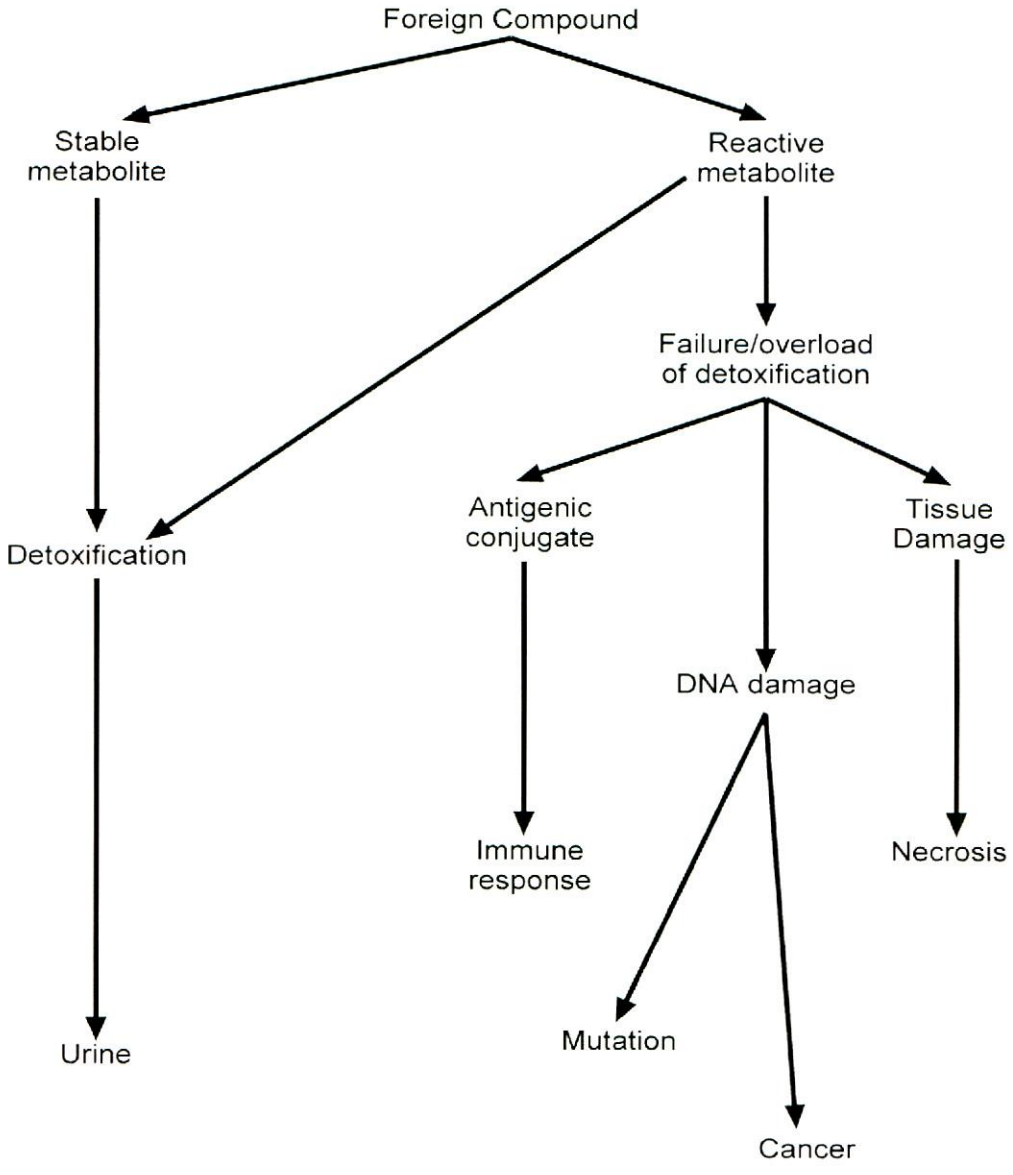


Fig. 5. The potential metabolism of xenobiotic/foreign compounds by phase I and phase II reactions.

2.4.1 Phase I reactions

Phase I reactions include oxidation, reduction, hydrolysis and hydration. The major types of oxidation reactions catalysed by P450 are divided into aromatic hydroxylation, heterocyclic hydroxylation, N-,S- and O-dealkylations, N-oxidations, N-hydroxylation, S-oxidation, desulphuration and deamination.

The principal function of P450 is the mono-oxygenation of various substrates which requires molecular oxygen and reducing equivalents from reduced nicotinamide adenine dinucleotides or dinucleotide phosphates (NADH or NADPH).

The P450-containing monooxygenase system primarily falls into two major classes, bacterial or mitochondrial (type I) and microsomal (type II) or alternatively can be classified according to the number of their protein components. The electron carrier proteins differ with subcellular location with both microsomal and mitochondrial P450s in eukaryotic cells using NADPH as electron donor while bacterial P450s use NADH.

The mitochondrial or bacterial P450 systems have 3 components, a FAD-containing flavoprotein (NADPH- or NADH-dependant reductase), an iron-sulphur protein and P450 (88) with the eukaryotic microsomal system containing two components, a flavoprotein containing both FAD and FMN (NADPH-cytochrome P450 reductase) and P450 (89). The P450 system consists of a group of enzymes located predominantly in the smooth endoplasmic reticulum of mammalian cells (90).

Upon subcellular fractionation of the cell, the P450 enzyme system is concentrated in the centrifugally-derived microsomal preparations (91). Although found in other tissues as well, the liver has the highest concentration of P450. The P450s have an iron-haem centre at their active site and in the reduced state these proteins form complexes with carbon monoxide to exhibit a maximum absorbance of light at 450 nm (92). Due to the flexibility in the ligand-binding pocket, each P450 has the capacity to oxidise a variety of different substrates. Exposure of P450s to a particular chemical and/or compound often leads to transcriptional activation of one or more P450 genes, resulting in the induction of enzymes involved in the metabolism and excretion of the ingested compound (91). Substances that may cause enhanced or induced activity of the P450 enzymes include caffeine, alcohol, dioxin, saturated fats, organophosphorous pesticides, paint fumes, sulfonamides, exhaust fumes and barbiturates (93). Based on sequence similarities, a standardized nomenclature has been adopted that categorises the individual P450s into respective families and subfamilies (94). Currently more than 700 P450s have been characterized, of which 50 are known to occur in humans. Table 3 summarizes the major P450 families and their isoforms.

P450s do not catalyse all Phase I reactions as other oxidative enzyme systems also exist. Epoxides may undergo hydration catalysed by the enzyme epoxide hydrolase located in the smooth endoplasmic reticulum near to the P450 system that produces the epoxides. This reaction can be regarded as a detoxification

reaction as the dihydrodiol products are less chemically reactive than the epoxide. However, the products, the trans diols, are further metabolised into more toxic metabolites (87). Another phase I reaction that may take place is reduction, utilising NADH or NADPH and is catalysed by either microsomal or cytosolic reductases and gut bacteria that possess reductases (Fig. 6). The most commonly found reaction is the reduction of nitro and azo groups. Reduction of nitro groups is an important route of biotransformation for e.g. nitrobenzene.

Table 3 Major cytochrome P450 families and their isozymes

Family	Subfamily	Isoform	Substrate activated	Induced by:
CYP1	CYP1A, 1B	CYP1A1	Polyaromatic hydrocarbons, e.g. benzo[a]pyrene	Cruciferous vegetables, cigarette smoke
		CYP1A2	Heterocyclic amines, Aromatic amines e.g. 2-AAF and AFB_1	Cruciferous vegetables, cigarette smoke
		CYP1B1	Metabolism of endogenous estrogens, biotransform heterocyclic amines from broiled meats	
CYP2	CYP2A, 2B, 2C, 2D, 2E	CYP2A1, A2, A3 (Expressed in rats)		
		CYP2A6, A7 (Expressed in humans)	Aflatoxin B_1, N-nitrosodiethyl-amine, nicotine	Barbiturates, Coumarin
		CYP2B1, B2(rats)	Amphetamines, benzodiazepines	Phenobarbital, barbiturates
		CYP2B6, (humans)	6-aminochrysene, Cyclophosphamide	Phenobarbital
		CYP2C8, C9, C19, C18, (humans)	S-mephenytoin, S-warfarin, non-steroidal anti-inflammatory agents	Rifampin
		CYP2E1	Acetaminophen,ethanol, acetone, benzene	Ethanol, acetone, isoniazid, starvation in animal models
CYP3	CYP3A	CYP3A4,	Aflatoxin B_1, acetaminophen, benzo[a]pyrene-7,8-dihydrodiol	Phenobarbital

Hydrolysis of esters and amides by carboxyl-esterases and amidases occurs in a variety of tissues but these enzymes are usually found in the cytosol of cells. Esterases can have amidase activity as well. Hydrazides and carbamates may be

hydrolysed by amidases (87) as amidases play an important role in the metabolic activation of certain drugs, e.g. isoniazid and phenacetin.

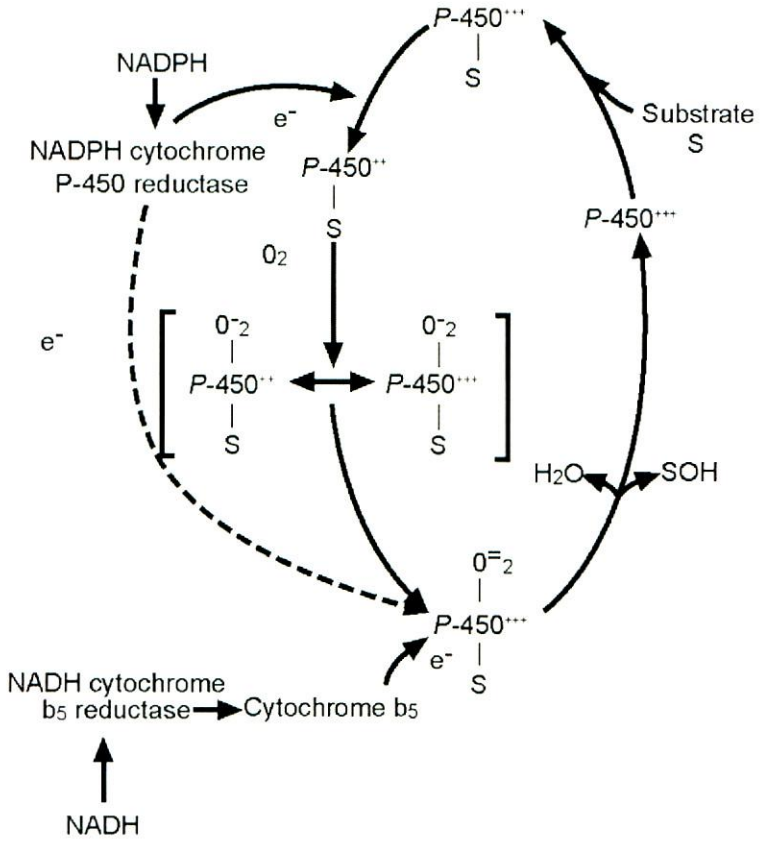


Fig. 6. The cytochrome P450 reduction reaction with NADPH and NADH as electron donors (95).

A number of compounds can also initiate redox cycling (cyclic reduction and re-oxidation) which may lead to the formation of reactive oxygen species [ROS] (96). These compounds are reduced by taking up an electron from NADPH to form a radical which is then oxidised by molecular oxygen. The ROS include hydrogen peroxide, hydroxyl radicals, singlet oxygen molecules and lipid peroxides and are all powerful oxidising agents that have the ability to initiate a wide range of responses including mutagenesis and carcinogenesis. Oxygen radicals interact with all cellular macromolecules, not only DNA. In particular, the polyunsaturated fatty acid residues of the phospholipids are extremely sensitive to oxidation (97). The resultant lipid hydroperoxides are relatively short lived as they are either reduced by glutathione peroxidases to non-reactive fatty acid alcohols or they react with metals to produce a

variety of reactive products, including epoxides and aldehydes. Malondialdehyde (MDA) and 4-hydroxynonenal (HNE) are the main products of lipid peroxidation (98). MDA reacts with DNA to form adducts to dC, dA, dG and was shown to be mutagenic in bacterial and mammalian cells and carcinogenic in rats (99,100).

The oxidant/antioxidant balance is highly regulated and oxidative stress induced by the overproduction of ROS lead to a disruption of cellular functions. The cell eludes injury caused by free radicals via a system of small molecule endogenous antioxidants, e.g. glutathione, vitamin E, C and enzymes e.g. superoxide dismutase (SOD), catalase and glutathione peroxidase.

2.4.2 Phase II reactions

Phase II reactions are known as conjugation reactions and involve the addition of a polar endogenous metabolite to a substrate molecule being either the parent compound and/or reactive metabolites resulting from phase I reactions (75). The polar moiety renders the substrate molecule more water-soluble facilitating excretion from the body via the kidneys or bile. The endogenous compounds include carbohydrate derivatives, amino acids, glutathione and sulphate. Phase II enzymes, such as uridine-5'-diphospho-α-D-glucuronic acid glucuronosyltransferases (UPD-GT), sulfotransferases and glutathione S-transferases (GSTs) catalyse these conjugation reactions and cofactors are essential as they provide the "donor" molecule.

Glucuronosyl transferases (UDP-GTs) are microsomal enzymes found mainly in the liver, intestinal mucosa and kidneys. The addition of glucuronic acid, a polar carbohydrate molecule, to hydroxyl, carboxylic acid, amino and thiol groups is a major route of phase II metabolism. Glucuronic acid is donated by a cofactor, UDP-glucuronic acid yielding an organic acid that can be readily excreted by specific transporters (87). This multigene family has been shown to participate in the conjugation of potentially harmful oxidative compounds (101). One class of compounds that contain candidates for the formation of oxidative derived mutagens is the aromatic and heterocyclic amines (Fig. 7). In the presence of the intermediate, N-hydroxy-2-AAF, glucuronidation is capable of providing sufficient protection against mutagenesis and is generally a detoxification reaction, but may occasionally be involved in increased toxicity of a xenobiotic (102).

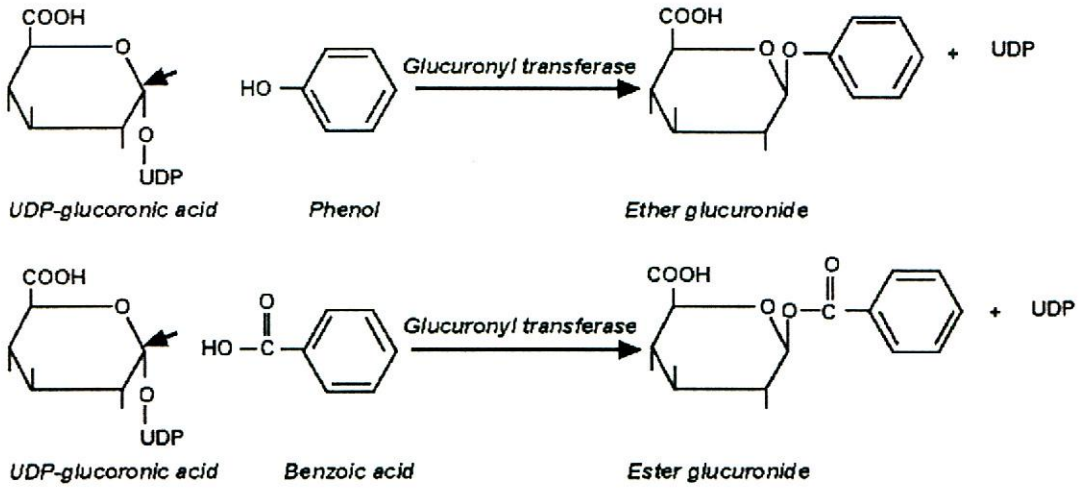


Fig. 7. Glucuronidation of phenol and benzoic acid to form ether and ester conjugates, respectively (95).

Sulfotransferases are cytosolic enzymes found mainly in the liver, but also in a variety of other tissues. They catalyse the conjugation of lipophilic chemicals with a sulfate moiety (Fig. 8) involving the cofactor, 3'-phosphoadenosine-5'-phosphosulfate [PAPS] (87). The water-soluble sulphate conjugate is an ester that is excreted in urine.

Sulfation is the main metabolic pathway for the elimination of steroid and thyroid hormones. Many factors may influence the activity of the sulfotransferases as a diet low in methionine and cysteine has been shown to reduce sulfation. Sulphate conjugation may also be involved in the metabolic activation of compounds such as the carcinogen 2-AAF (103).

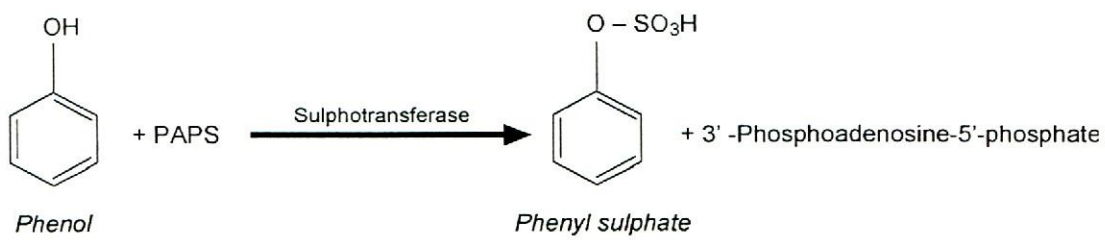


Fig. 8. Conjugation of an aromatic hydroxyl group with sulphate (95).

The GST enzymes catalyse the reaction of electrophilic compounds with glutathione (GSH) rendering the products more water-soluble (104). The cytosolic GST family is grouped into 3 classes of cytosolic isoenzymes, alpha (α), mu (μ) and pi (π) and a microsomal membrane bound (theta) form (105). This conjugation is irreversible and an important phase II reaction as it is involved in the active removal of reactive intermediates. Detoxification of the ultimate carcinogen, AFB_1-8,9-epoxide, through GSH conjugation is a key mechanisms in reducing AFB_1-induced hepatocarcinogenesis in rats (106). After the addition of GSH and the subsequent removal of two amino acids, the cysteine conjugate is acetylated to yield the N-acetylcysteine conjugate (Fig. 9).

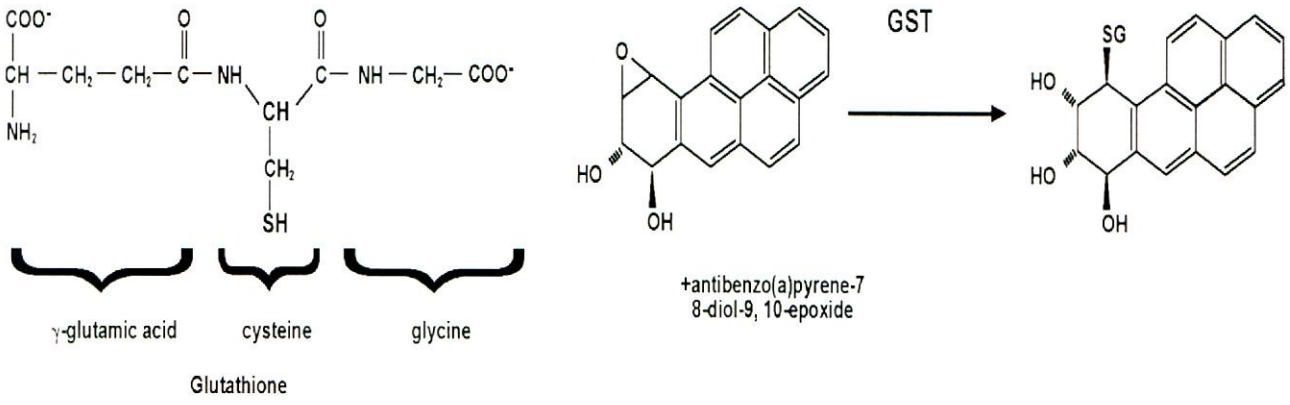


Fig. 9. Glutathione conjugation of benzo[a]pyrene-7,8-diol-9,10-epoxide (107).

As a cellular nucleophile, GSH may react chemically via the sulfhydryl groups that attack electrophylic sites. GSH is a sulphur-containing tripeptide consisting of the amino acids γ-glutamic acid, cysteine and glycine (Glu-Cys-Gly) and found in many mammalian tissues, specifically the liver. Glutathione exists in both a reduced state (GSH) and an oxidised state (GSSG) and GSSG is usually less than 1% of the GSH content. In healthy cells, normal cellular glutathione homeostasis is maintained through regeneration from GSSG via glutathione reductase (Fig. 10) and/or through GSH uptake from exogenous sources (108). Reduced glutathione is vital to a broad range of cellular functions including quenching of ROS and free radicals, detoxification of xenobiotics and the maintenance of a reduced cellular status.

Acetylation involves an activated cofactor, acetyl coenzyme A (acetyl CoA) and may yield a compound that is less water-soluble e.g. when an amine is converted into an amide. The acetyl transferases involved in acetylation reactions occur in the cytosolic fraction of liver, gastric mucosa and white blood cells. Acetylation is an important route of metabolism for aromatic amino compounds, sulfonamides, hydrazines and hydrazides (87). Amino acid conjugation is the second type of acylation reaction in which the xenobiotic compound is metabolized resulting in the formation of an amide bond to an amino acid. The reaction is catalysed by amino acid acyl transferase enzymes and is dependent on thiamine, pantothenic acid and vitamin C. Glycine is the most common conjugating amino acid in mammals.

Methylation is a common, but generally minor pathway of xenobiotic metabolism and involves the transfer of methyl groups to substrate molecules. It differs from most other phase II reactions because it generally decreases the water solubility of a compound. Methylation is mediated by three groups of enzymes, the O, N, and S-methyltransferases.

Most of the methyl groups utilised in the phase II reactions originate from the cofactor S-adenosylmethionine (SAM) that is synthesized from the amino acid methionine, a reaction requiring choline, vitamin B12 and folic acid. Hormones such as estrogens are inactivated through methylation using SAM (87).

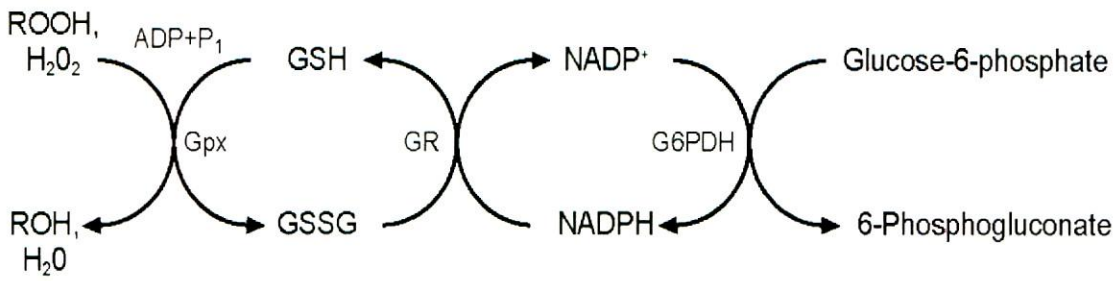


Fig. 10. The glutathione cycle (108).

2.4.3 Phase III reactions

A possible phase III detoxification system with antiporter activity (Fig.11) has recently been described (109). The antiporter is an energy-dependent efflux pump, transporting xenobiotics out of the cell and resulting in a decreased intracellular

3. Cancer chemoprevention

"He is a better physician that keeps diseases off us, than he that cures them being on us; prevention is so much better than healing because it saves the labour of being sick."

Thomas Adams, 1618

"Chemoprevention is the use of pharmacological or natural agents that inhibit the development of invasive cancer either by blocking the DNA damage that initiates carcinogenesis or by arresting or reversing the progression of premalignant cells in which such damage has already occurred. The word chemoprevention includes prevention of initiation, promotion and progression of carcinogenesis to cancer."

Michael Sporn, 1976

3.1 General

Controlling cancer involves the prevention of a complex series of genetic and epigenetic events that result in invasive and metastasised malignancy. The "chemo-prevention strategy" was founded in the mid 1970s by Michael B. Sporn (116) who defined it as using one or several chemical compounds (natural or synthetic) to prevent, inhibit or reverse the process of cancer development.

The National Cancer Institute (NCI) in the United States of America has made chemoprevention research a top priority and more than 400 compounds are currently under investigation as potential chemopreventive agents (117). These agents can either prevent and/or stop mutations or the processes leading to excessive cell replication that may lead to cancer development of genetically damaged cells. Ideally such agents should be inexpensive, easily administered, highly effective, showing minimal short term and no long-term toxicity (117). Chemopreventive compounds generally exhibit three major activities: 1) inhibiting the uptake, formation or activation of carcinogens by enhancing the deactivation of ultimate carcinogen thereby preventing DNA-carcinogen adduct formation; 2) antioxidant scavenging of reactive electrophiles, oxygen radicals or inhibiting the formation of eicosanoids; and 3) interfering with cell proliferation of transformed cells to modulate signal transduction pathways, inducing apoptosis or inhibiting angiogenesis (118). Identification of such chemopreventive compounds,

biomarkers of risk, biomarkers of efficacy and suitable cohorts for clinical intervention are critical for the progression of chemoprevention. Successful implementation of chemopreventive strategies also depends on the mechanistic understanding of carcinogenesis at molecular, cellular and tissue level.

The credibility of chemoprevention as a serious and practical approach to the control of cancer has been enhanced by the publication of clinical trials. Tamoxifen, raloxifene and 4-hydroxyphenylretinamide (synthetic retinoid) have been shown to be effective agents for prevention of breast cancer in women with varying degrees of risk (119-121).

3.2 Human intervention trials

Potential chemopreventive compounds have to pass through several testing phases in humans before they are rendered "safe and effective" to be prescribed to the population at large (122). During these phases the effectiveness, toxicity and possible side effects of the compounds are determined, as preclinical toxicity screening in animals does not always accurately reflect toxicity in humans. The individuals targeted for chemoprevention trials include the following persons: those with lifestyle risks, such as smoking or high fat diet, persons with a family history of cancer, persons at high risk of cancer development because of the presence of precancerous lesions and persons at risk of developing a second primary cancer.

Performing phase 1 trials is the first step in evaluating new compounds for toxicity and to determine the dose-related safety and pharmacokinetics in normal subjects or subjects with pre-malignant lesions (123). This dose escalation scheme in phase 1 trials determines the dosages for phase 2 trials. The latter usually involves 25-100 people, lasts less than 12 months and also focuses on possible side effects of the compounds in humans. In phase 2 trials the efficacy of the compound against intermediate endpoints of cancer risks (e.g. precancerous lesions) is assessed and involves 100-1000 human subjects and last 1-5 years. The majority of phase 3 trials target the reduction of precancerous lesions and therefore cancer incidence as primary endpoints, usually last 5-10 yrs and involves 1000-10 000 human subjects. These studies also focus specifically on long term side effects the chemopreventive agent might have (123).

Currently the NCI does not recommend supplements of vitamins, minerals or any other compound for the prevention of certain cancer(s) as many short and long term effects of these possible chemopreventive compounds are still unknown. Recently, many studies produced contradictory results. The outcome of three randomized β-carotene intervention trials, started in the 1980s, had lowered the enthusiasm for using β-carotene in chemoprevention studies against lung, oral cavity and pharyngeal cancers (124-126). In the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (ATBC), a double blind trial was used to evaluate high daily dosages of vitamin E (α-tocopherol), β-carotene or a combination of both on reducing the incidence rates of particularly lung cancer or other cancers among male smokers (127). Alpha-tocopherol had no effect on the incidence of lung cancer, while the men who received β–carotene presented with a higher incidence (>16%) of lung cancer. This trial was the first to support the possibility that β-carotene may also be harmful. Since then, two other big intervention trials using β-carotene were conducted. The Beta-Carotene and Retinol Efficacy trial (CARET), a multicenter randomized chemoprevention trial designed to test a combined lung cancer prevention agent in heavy smokers and asbestos workers, reported a 28% increase in lung cancer and 17% more deaths in the active intervention group receiving a daily dose of 30 mg of β-carotene and 25,000 IU of retinyl palmitate (125,128). On the other hand, the strongest evidence of a benefit of vitamin and mineral supplementation arises from the large Linxian general population trial that indicated patients receiving a combination of β-carotene, Vitamin E and Vitamin A showed a significant reduction in stomach cancer mortality (129). The process of chemoprevention is complex, but the potential to increase the general health of a population and reduce cancer incidence rates in high risk individuals does exist (130). Improved knowledge of the level of these chemopeventive agents, their metabolites that prevent cancer in rodent models and levels achievable in human tissue are needed (131).

3.3 Animal studies and chemoprevention

Animal models play a significant role in cancer prevention investigations. Hypothesis resulting from human epidemiological studies can be tested in animal models, as they not only provide a system to identify potential chemopreventive agents but also help to elucidate the protective mechanisms involved. These animal models are also used as a tool to establish and/or verify the use of biomarkers for specific cancer types and to

determine non-toxic dose ranges. Potential chemopreventive compounds are screened in a series of preclinical tests, including *in vitro* antioxidant, antimutagenicity assays and *in vivo* experimental animal cancer models to monitor any protective effect. Data generated from these investigations as well as from animal toxicology tests to evaluate drug safety and pharmacokinetic characterisation, are utilized to identify compounds to be used in human trials. The development of transgenic rodents has been of great advantage in studying the prevention of oestrogen receptor (ER)-negative breast cancer in addition to the well established ER-positive breast cancer rat models (132).

The evaluation of a chemopreventive agent in a relatively small number of animals where the controls show a 100% tumour incidence within a short latency period is suggested to be the preferred method for laboratory studies (133). However, the latency period in humans can be up to 20 years and the incidence of invasive cancer is low, thus chemopreventive agents that double the latency period for human carcinogenesis would have a great impact on increasing the quality of life for millions of people. Over 2000 compounds of at least 20 structural and pharmacological activity classes have been shown to possess chemopreventive activity, but less than 10% of these compounds have been subjected to *in vivo* testing using animal carcinogenesis models. However, it is still unclear whether the effects in animals can be readily extrapolated to humans as the dose used is often higher in animal models. Certain agents such as phytochemicals are fermented by colonic bacteria, altering the compound absorbed from the gut from that which was originally consumed. The original phytochemical may, in fact, not be detectable in the blood at all (134).

3.4 Phytochemicals and chemoprevention

There is strong scientific evidence, both epidemiologically and experimentally, that modifications in diet such as increased intake of fruits and vegetables, can prevent a range of human disease, including cancers (135,136). Vegetables and fruits are a rich source of a variety of nutrients, including vitamins, trace elements, dietary fibre and many other classes of biologically active compounds (Table 4). The cancer inhibitory action of dietary components derived from plants, referred to as phytochemicals, has been confirmed in various animal cancer and/or carcinogenesis models (137,138). Studies in cell culture systems have provided a wealth of information on the mechanisms by which a diet high in fruits and vegetables may lower the risk for chronic

diseases in human. In recent years, the isolation, identification, characterization and quantification of phytochemicals and their potential health benefits to humans have become an important area of scientific research.

Table 4 Dietary plant sources of possible chemopreventive compounds

Phytochemicals (non-nutrient chemopreventers)	Dietary source
Fiber	Cereals (grains, fruits, vegetables)
Carotenoids	Yellow/orange vegetables, fruits, dark leafy vegetables
Allium compounds	Onion, garlic, chives, leeks
Dithiolthiones/glucosinolates	Cruciferous vegetables
Isothiocyanates	Cruciferous vegetables
Terpenoids	Oil of citrus peel
Phytoestrogens	Cereals, pulses, sorghum, millets, soyabeans, fruits, berries
Protease inhibitors	Cereals, barley, wheat, oats, rye, soyabeans, kidney beans, chick peas
Phytic acid	Cereals, nuts, seeds, sesame seeds, lima beans, peanuts, soyabeans
Flavonoids	Fruits, vegetables, tea
Phenolic compounds	Fruits vegetables, tea
Plant sterols	Vegetables
Saponins	Soyabeans, yams, colacasia

Approximately 30 classes of phytochemicals including flavonoids, isoflavonoids, isothiocyanates, indoles, dithiolthiones, coumarins, isoprenoids and organosulphides have been isolated and tested for their potentially anticancer activity (130,139).

3.4.1 Flavonoids and tannins

Flavonoids are low molecular weight compounds found in all vascular plants, e.g. fruits, vegetables, nuts, seeds, herbs, spices, stems, flowers, red wine as well as tea (140) and are recognised as pigments responsible for the many shades of yellow, orange and red. These phytochemicals cannot be synthesized in the human body (141). More than 5000 different flavonoids have been described and have been classified into more than 10 chemical groups (141,142). The structure of flavonoids is based on the flavonoid nucleus (Fig. 11) consisting of three phenolic rings, A being the benzene ring which is condensed with C, a six-member ring which carries at position 2 a phenyl benzene ring, B.

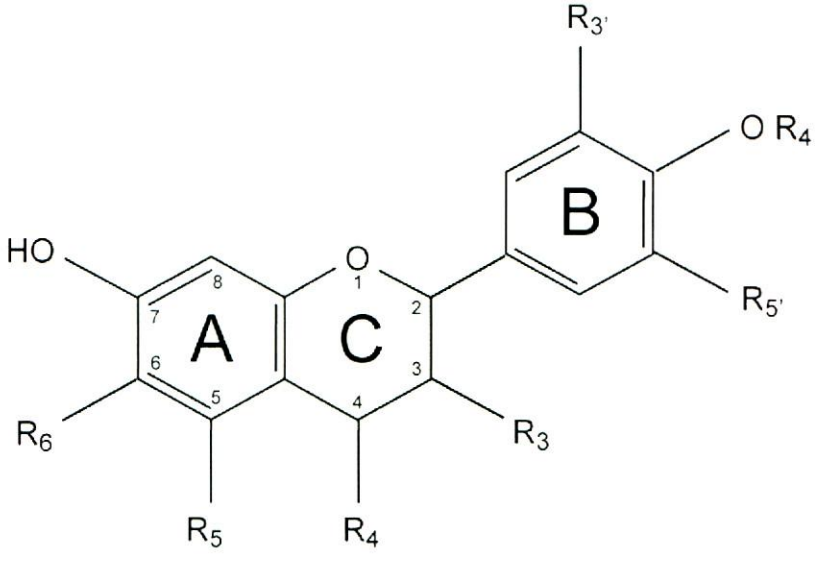


Fig. 11. The generic structure and numbering pattern for common food flavonoids (143).

The flavonoids are classified into subgroups according to substitution patterns in the A, B and C rings and include flavanols, flavanones, flavonols, flavones, isoflavones and anthocyanidins [Fig. 12] (144,145). Flavonoids primarily exist as 3-O-glycosides (flavonoid molecules linked to sugar moieties) and polymers in food, as glycosylation increases the polarity of the flavonoid molecule necessary for storage in plant cell vacuoles (146-148).

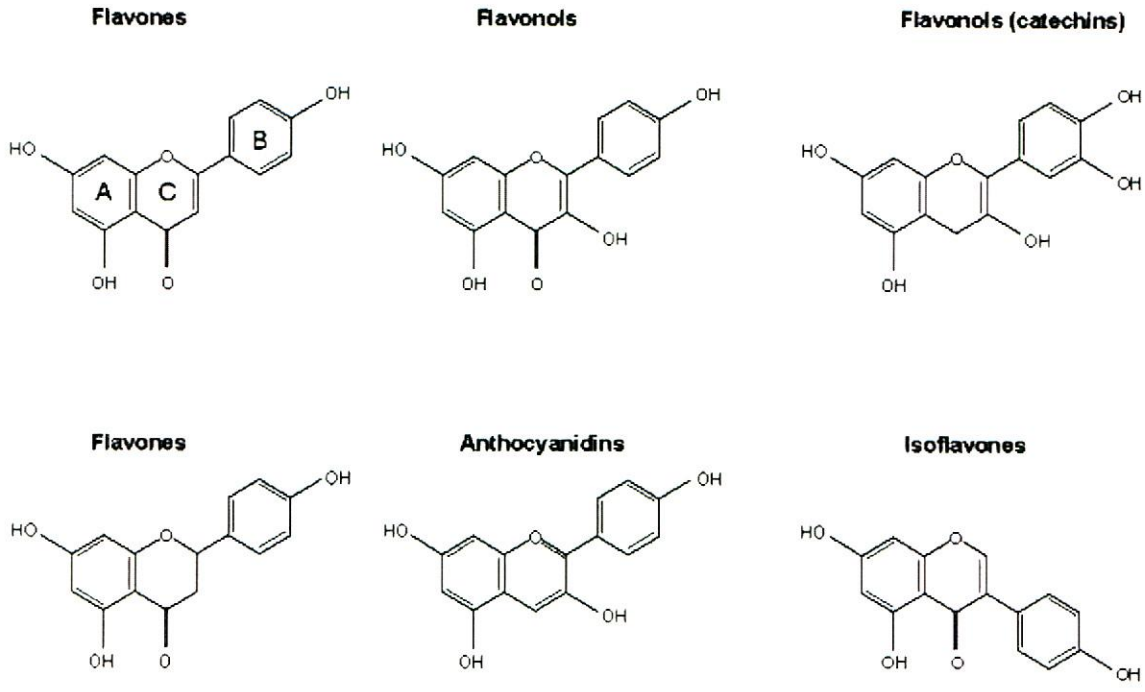


Fig. 12. The structures of the major classes of flavonoids (143).

Various dietary sources of flavonoids are shown in Table 4 and it is important to note that the flavonoid content is strongly influenced by variation in plant type, growth, season, climate, degree of ripeness, food preparation and food processing (148). The dietary intake of flavonoids (based on only a subset of the flavonoids, quercetin, kaempferol, myricetin, apigenin and luteolin) in various populations has yielded estimates of 3-68 mg, but tea drinkers may have an intake up to 430 mg per day based on the intake of 3.3 cups of tea per day (149,150).

Tannins are natural occurring plant polyphenols and are made up of flavonoid units (151) responsible for the astringent or bitter taste of plant products such as wine, unripe fruits and various teas. Tannins can be divided into three types, the non-hydrolysable or condensed tannins that are polymerised forms of flavonoids, the hydrolysable tannins that are gallic acid esters of simple sugars like glucose and the derived tannins that are complex compounds formed primarily during the processing of plants (152). The condensed tannins are also referred to as proanthocyanidins (153).

Table 4 Dietary sources of subclasses of flavonoids

Class	Flavonoid	Dietary source
Flavonols	Quercetin, Myricetin, Kaempherol	Onions, Apple skin, Tea, Berries, Black grapes
Flavones	Rutin, Luteolin, Apigenin, Chrysin	Onions, Apple skin , Berries, Black grapes, Tea, Broccoli, Celery, Parsley, Lemon, Olive
Flavanols	Catechins, Epicatechins, Epigallocatechins, Epigallocatchin gallates, Epicatechin gallates	Black grapes, Teas
Flavanone	Naringenin, Hesperidin, Hesperetin, Taxifolin, Narirutin	Citrus fruit, Orange juice, Grape fruit
Anthocyanins	Cyanidin, Oenin, Delphinidin	Black grapes, Grapes, Raspberries, Strawberries, Aubergine skin
Isoflavones	Genistein, Daidzein, Biochanin A, Formononetin, Coumesterol	Soybeans, Black beans, Green split peas, Clover sprouts, Lima beans, Sunflower seeds

Adapted from Peterson and Dwyer (141) and Rice-Evans *et al.* (146).

Tannins have the ability to form complexes with proteins, starch and digestive enzymes and reduce the nutritional value of foods. Tannin-protein interactions are mostly based on the formation of hydrophobic and hydrogen bonds. The phenolic group of tannin is an excellent hydrogen donor forming strong hydrogen bonds with the carboxylic groups available on the protein (154). Of importance to oolong and black teas are the flavanol-derived theaflavins. These tannins reduce the bioavailability of non-heme iron when tea is consumed with meals by forming insoluble complexes within the gastrointestinal tract, rendering the iron unavailable for absorption (155,156). Other condensed type of tannins have also been reported to inhibit digestive enzymes, including pectinase, amylase, lipases, proteolytic enzymes, β-galactosidase, cellulase and those microbial enzymes involved in fermenting cereal grains (153).

3.4.1.1 Absorption, metabolism and bioavailability of flavonoids

The absorption and subsequent distribution, metabolism and excretion of flavonoids in human and animal studies are of great importance to better understand their health benefits. Bioavailability is defined as the amount or percentage of an ingested nutrient/compound that is absorbed and thus available for metabolic use (157). Various factors may influence the bioavailability such as isomeric form, processing methods, type of compound and matrices surrounding the compounds. Many studies have reviewed the

bioavailability and health effects of dietary flavonols in humans indicating that quercetin glycosides were more readily absorbed than the pure aglycone suggesting that the glycosides may be absorbed via the intestinal sugar uptake route (158). However, the question still remains as to which form is actually absorbed from the gut, the aglycone, the glycoside or both. The hydrophobic flavonoid aglycones are transported across membranes by passive diffusion, while with the addition of a glycoside moiety, the flavonoid molecule becomes more hydrophilic, thereby reducing the possibility of passive transport. A theory has been proposed whereby the flavonoids are absorbed via active transport, but this has not been proven (159). A study by Walle *et al.* (160) could not detect any quercetin glucosides in the ileostomy fluid, but identified substantial amounts of the aglycone form. It was suggested that quercetin glucosides are efficiently hydrolysed to the aglycone in the small intestine by β-glucosidases and then absorbed. Further evidence is required to establish the possible role of sugar transporters in flavonoid glycoside uptake.

Deglycosylation of flavonoid glycosides has been proposed to be the first step in the metabolism during the absorption process whereafter the liver seems to be the main organ involved although the intestinal mucosa and/or kidneys cannot be ignored. Flavonoids are either methylated, sulphated, glucuronidated or hydroxylated (144) after absorption. Conjugation with glucuronic acid and/or sulphate seems to be the most common type of metabolic reaction in the liver to increase the molecule's polarity and facilitates its excretion (141,161) via the urine or bile. Flavonoids that are not absorbed in the small intestine can be metabolised by colonic microflora into aglycones and phenolic acids. In the presence of intestinal microflora ring scission may occur, accounting for the subsequent demethylation and dehydroxylation of the involved phenolic compounds. Intestinal bacteria also possess glycosidases capable of cleaving sugar residues resulting in aglycones that can then either be absorbed or further metabolized to more simple phenolic acids (162,163). Evidence is increasing to confirm that human intestinal bacteria have a selection of hydrolytic enzymes capable of degrading various dietary flavonoid glycoside compounds.

Improved analytical methods have enabled the detection of flavonoid concentrations as low as 10 nM in human plasma samples (164). In a study conducted by Das (165) the oral administration of 83 mg/kg bodyweight of (+)-catechin to humans resulted in rapid absorption, metabolism and excretion of the flavonoid within 24 hr.

Using more updated analytical techniques, peak plasma levels of the flavonoid, quercetin, were measured after 2.9 h of ingesting fried onions containing quercetin glycosides (166). A study in animals showed that rats fed a 0.2% quercetin diet exhibited an increased plasma antioxidant status when compared with control animals (167).

Adverse reactions to flavonoids in humans appear to be minimal (168) and safe with a wide range of biochemical and pharmacological activities. These findings imply their possible role as health promoting and disease preventing dietary supplements. Although flavonoids exhibit various biological activities as mentioned above, for the scope of this review, only those properties that may play a role in cancer prevention will be discussed.

3.4.1.2 Biological properties of flavonoids

The mechanisms of action of most chemopreventive agents are complex and classified according to the site of action or by the specific type of action. It appears that the flavonoids act primarily as antioxidants, antimutagens, immunomodulators and anticarcinogens (169-172).

(i) Antioxidant activity. Oxidative stress may lead to the damage of many biological molecules such as structural proteins, membrane lipids, DNA, and RNA. An effective antioxidant is defined as a compound, that when present at low concentrations relative to an oxidizable substrate, can suppress, delay or prevent oxidation of these macromolecules (173). The antioxidant properties of flavonoids have been investigated since the mid 1960s with quercetin and rutin receiving the most attention (174). Flavonoids act as natural antioxidants by functioning as metal chelators and reducing agents, scavengers of reactive oxygen species, chain-breaking agents, quenchers of the formation of singlet oxygen and stabilization of endogenous antioxidants (146,147). The antioxidant potential of flavonoids is predicted by the chemical structure and the relative positions of the hydroxyl groups (175).

Human cells are constantly exposed to reactive oxygen species (ROS) and increased exposure may cause damage of cellular molecules leading to the development of cancer and cardiovascular diseases. The term ROS is used for oxygen-centered radicals e.g. superoxide ($O_2^{\cdot}$) and hydroxyl ($^{\cdot}OH$) as well as non-radical species derived

from oxygen, e.g. hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hypochlorous acid (HOCl). These radicals also have the ability to initiate and promote carcinogenesis (145). Both enzymatic as well as non-enzymatic systems exist in the cell to detoxify ROS. Endogenous enzymatic defences include superoxide dismutase, catalase, glutathione peroxidase and glutathione-regenerating systems, but are not completely efficient especially under certain diseases conditions (176). Other endogenous and exogenous antioxidants are needed to assist in the body's defences against oxidative damage. Flavonoids are known to be effective scavengers of ROS and other free radicals, as epidemiological studies identified inverse associations between flavonoid consumption and risk for cancer and cardiovascular disease (177-181). Ishige *et al.* (181) showed that flavonoids protect cells from oxidative stress by altering glutathione (GSH) metabolism, resulting in a 30-80% increase in the basal level of total GSH. Lipid peroxidation is also of biological importance, whether being a primary event produced by oxidative stress or a consequence of tissue damage, as products originating from dying cells can have a cancer promoting effect (98). Flavonoids such as quercetin have been reported to suppress lipid peroxidation in several biological systems, e.g. mytochondria, microsomes, and erythrocytes by acting as strong oxygen radical scavengers (145).

Another study indicated flavonoids to exert prooxidant activities. The presence of EDTA (pH 7.4) containing ferric iron (Fe^{3+}) and 100μM quercetin enhanced hydroxyl radical formation from hydrogen peroxide (H_2O_2) in the deoxyribose assay (182). From this study and various others, the prooxidant activity appears to require the presence of Fe^{3+} and high flavonoid concentrations and has been suggested that both are not likely to occur in physiological systems.

(ii) Anticarcinogenic activity. The relationship of flavonoid intake and cancer prevention has been well documented (183). Various studies indicated the antimutagenic and/or anticarcinogenic activity of different flavonoids when tested in bacterial mutagenicity assays and carcinogenesis models (184). Quercetin inhibits the mutagenic activity of benzo[a]pyrene (BP) in bacterial mutagenicity studies as well as BP-induced DNA damage in mice colonic epithelial cells (185,186). Topical application of quercetin to mouse skin has been reported to protect mice against DMBA-, BP-, N-methyl-N-nitrosourea-, and BP-7,8-dihydrodiol- and IQ-epoxide-induced skin tumourigenesis (61,187). When quercetin was fed to rats in their diet over a period of weeks, the number of hepatocellular carcinomas in rats treated with the liver tumour promoter phenobarbital

was significantly decreased (188). Several possible mechanisms exist in the inhibition of carcinogenesis by flavonoids and include: 1) inhibiting the metabolic activation of the carcinogen to its reactive intermediate; 2) inducing the enzymes involved in the detoxification of the ultimate carcinogen; and 3) scavenging of free radicals, thereby preventing their interaction with cellular targets (115). Inhibition of the isoenzyme CYP1A family by quercetin, myricetin and anthraflavic acid has been shown, and suggested to be, an inhibitory mechanism as discussed above (189,190). However, induction of the P450 system by natural and/or synthetic flavonoids has also been reported (191,192). Controversies in the literature do exist with regard to compounds operating via the induction of P450 enzymes, as some scientists suggest that it increases susceptibility to some classes of carcinogens and that compounds inducing the conjugating phase II enzymes are less likely to be potentially hazardous. Siess *et al.* (176) studied the effect of flavone, flavanone and tangeretin on the induction of various drug metabolising enzymes in rats fed a diet containing 20-2000 ppm. The flavone and flavanone showed a dose dependent induction of GST as well as UDP-GT, while tangeretin showed a slight stimulating effect on UDP-GT. The effect of various flavonoids in the diet of rats on AFB_1-induced hepatocarcinogenesis was investigated in a subsequent study (193). The non polar flavonoid, flavanone, exhibited an anti-initiating effect while delaying the promotion phase. Tangeretin and quercetin induced a less protective and no protective effect, respectively. The induction of phase II enzymes (UDP-GT and GST), increased formation of AFB_1-GSH conjugates in the liver cytosol as well as a significant decrease in AFB_1-DNA adduct formation by the flavonoid attributed to the protective effects obtained in this study.

(iii) Other diverse activities. Various flavonoids, e.g. hesperidin, luteolin and quercetin exhibit anti-inflammatory activity by inhibiting cycloxygenase-2 (COX2) and inducible nitric oxide synthase [iNOS] (145). Nitric oxide ($NO^{\cdot}$) is a free radical with a single unpaired electron, not very reactive, but when overly produced is involved in chronic inflammatory diseases and initiation of lipid peroxidation (194). In the presence of molecular oxygen (O_2), NO autooxidizes to produce a variety of nitrogen oxides, referred to nitrosating species, such as nitrogen dioxide, nitrite and dinitrogen trioxide. Nitrosation of secondary aromatic amines may produce potentially carcinogenic nitrosamines. These promote mutagenesis and carcinogenesis via their ability to alkylate specific sites in DNA. In the absence of superoxide (O_2^{-}), NO will nitrosate certain primary and secondary amines forming potentially mutagenic nitrosamine intermediates promoting the nitrosative

deamination of DNA bases, while in the presence of both NO and superoxide, N-nitrosation may be suppressed but oxidation reactions may dominate (195). Depending upon the fluxes of each radical (NO or O_2^-) either nitrosation or oxidation will dominate. Flavonoids suppress the oxidation of NO to nitrite and scavenge the reactive nitrogen species (RNS) formed from NO such as peroxynitrite (196,197). NO metabolism in the vascular endothelium appears to be an important target explaining the beneficial effects of dietary flavonoids.

Flavonoids also affect cell signalling and cell cycle progression via induction of cell cycle arrest and apoptosis (198). Disregulation of cell proliferation is implicated in an increased susceptibility to neoplasia, thus cancer prevention is generally associated with inhibition, reversion or retardation of cellular hyperproliferation. Many flavonoids and isoflavonoids inhibit cellular proliferation in various human cancer cell lines (199). Tumour cell lines exposed to quercetin showed appropriate morphological changes, DNA laddering and condensation of nuclear chromatin, characteristic of apoptosis (199,200). In addition, quercetin also exerts growth-inhibitory effects on several tumour cell lines *in vitro* (200) which may be due to its interaction with nuclear type II oestrogen binding sites (EBS) as the relative binding affinity correlated well with growth inhibition (201,202). Certain checkpoints of the cell cycle in cultured cancer cell lines, G1/S and G2/M have been shown to be disrupted by flavonoids such as genistein, quercetin, silymarin and apigenin. Although antiproliferative effects of flavonoids have been well established *in vitro*, relatively little data exists on the *in vivo* antiproliferative effects.

Recently, Zang *et al.* (203) showed that flavonoids interact with the aryl hydrocarbon receptor (AhR) *in vitro*. This receptor is the same through which 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a toxic halogenated aromatic industrial by-product, exerts its toxic and mutagenic effects. Certain flavonoids were shown to be AhR agonists, mimicking TCDD's action while other flavonoids, e.g. luteolin were antagonists, blocking the stimulation of the AhR by TCDD. This study suggested that the effect of dietary flavonoids should be taken into account when assessing TCDD-induced biochemical and toxic responses in humans, but it is likely that the dietary intakes of most phytochemicals would be below the levels required for an AhR agonist, based on the *in vitro* cell culture studies (203).

3.5 Summary

There is a great variety of dietary flavonoids and it seems unlikely that one particular compound is responsible for all the associations described between plant components and cancer prevention. Although multiple biological actions for dietary flavonoids against cancer have been identified, it is not clear whether these effects would also be present at physiological concentrations and/or for the flavonoid metabolites. More data is needed before any of the possible inverse associations between flavonoid intake and various cancers be used to support specific health recommendations (204). A better understanding of synergisms and antagonisms between various flavonoids present in our daily diet is needed.

Chemopreventive compounds mainly act as enzyme modulators, antioxidants, inhibitors of carcinogen-adduct formation, inhibitors of oncogene activation or modulators of signalling cascades associated with carcinogenesis (204). These compounds are not only expected to interfere with the carcinogenesis process, but also with the normal homeostatic processes in the body. Side effects may include enhancement of carcinogenicity under certain conditions. Evaluation of potential toxicological effects of these compounds should be thorough and elucidation of the mechanisms involved is imperative.

4. Tea and herbal beverages as possible tools for chemoprevention

"Bodhidharma, the first Zen Buddhist legend around the 6th century A.D. tried to keep himself awake during meditation by cutting off his eyelids. The eyelids grew as the first tea plants and the tea has been used since to fend off sleep and clear the soul (8). The Chinese emperor, Shen Nung, while on a journey, had a few leaves from a wild tea tree fall into hot water producing a yellow brownish beverage that he tasted and liked. He declared it gave vigour of body, contentment of mind and determination of purpose. Gautama Buddha is said to have discovered tea when a leaf fell into his cup while meditating in the garden (http://www.nationmaster.com/encyclopedia/tea)."

Herbal teas are not regarded as true teas as they are not derived from *Camellia sinensis,* but from other sources such as blossoms, leaves, stems and fruits of various other plants (205). Herbal teas are more properly referred to as infusions or tisane. In addition to being a safe, enjoyable and inexpensive beverage, tea also provides a natural source of compounds that protect against various diseases (206-209). During the past 7-8 years research from across the world yielded increasing scientific evidence of the health benefits of tea to animal and man.

4.1 *Camellia sinensis*

4.1.1 History and processing

Tea derived from the evergreen shrub, *Camellia sinensis* belonging to the Theaceae family, is used as a daily beverage worldwide and is second only to water in popularity. Tea is usually prepared by infusing green or black tea leaves in a proportion of 1 g leaf to 100 mL freshly boiled water for approximately 3 minutes. Tea has been consumed socially and habitually by humans for more than 4000 years and the first accepted reference is found in a Chinese dictionary from 350 A.D. stating the beverage was used as a medicine for various illnesses (210). Tea was first cultivated in China and in the late 12th century, Japanese Zen priest, Eisai, brought the seeds of *Camellia sinensis* to Japan (208). Tea is a perennial crop, with the bushes reaching maturity after 7 years and maintaining steady yields for up to 100 years (211). Tea can be cultivated in many regions with a high humidity, fair temperature and acidic soils from sea level to mountainous areas (212). Based on the leaf features, two intra-specific varieties have been identified, the China variety, referred to as *C. sinensis* var. *sinensis* (Fig. 13) and the Assam variety referred to as *C. sinensis* var. *assamica* (213).

Fig.13. *Camellia sinensis* plantation and picking of tea leaves (http://www.dal.ca/~dp/vigs/tea.html).

The China variety is a slow growing, dwarf shrub with small, narrow serrated and dark green leaves, while the Assam variety is a quick growing tree with large, broad, mostly non-serrated and light green leaves (211). Commercial cultivation has now expanded and the major exporting countries include China, India, Kenya, Sri Lanka, Argentina, Indonesia and Japan. India is the world's largest tea producing as well as tea consuming country. In 1996 the annual world tea production was 2 610 569 metric tons (214). Americans, Europeans, Africans and Indians typically prefer black tea, while Japanese and Northern Chinese prefer green tea, with oolong and paochong tea (semi fermented) favoured in Taiwan and parts of China (215).

The teas differ only according to the different degree of "fermentation" or chemical oxidation involved in their manufacture. Black tea (Fig. 14) is made by maceration of fresh tea leaves after withering to effect "fermentation" before drying. The

crushing and maceration of the tea leaves partially destroys the cell structure to allow enzymatic oxidation of the flavanols in the presence of oxygen (216). During the "fermentation", when endogenous enzymes catalyse aerobic oxidation, oligomeric flavanols e.g. theaflavins and thearubigins are formed to provide the characteristic flavour and colour of black tea (215). After "fermentation" the teas are fired by hot air dryers to stop the enzymatic fermentation and reduce the moisture content to about 3%. During the firing process the flavour and aroma is further developed (216) where after it is sorted into the appropriate grades. Green tea (Fig.14) is prepared from fresh leaves that are rapidly subjected to heat treatment after harvest to prevent any oxidative fermentation. This is done by either steaming (Japanese style) the leaves or roasting them in a pan referred to as Chinese style (217). The leaves are then pressed, rolled, dried and sorted into various grades. The composition of the teas also varies with the species, season, age of the leaf, climate and horticultural practises (215,218).

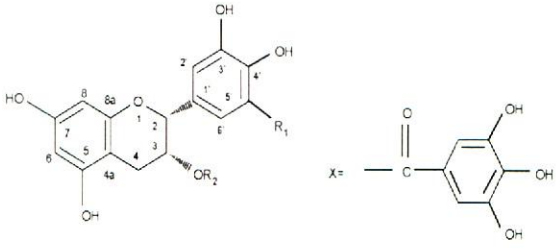
Fig. 14. Commercially available black (left) and green tea (right).

Another tea that has received little attention for its health benefits is white tea. White tea is amongst the rarest and most expensive varieties of tea and is exclusively produced in China, mainly in the Fukien province and represents the least processed form of the teas. This tea is made from the new growth buds shielded from the sunlight to prevent the formation of chlorophyll. Little data is available on the phenolic constituents and biological properties of this tea (219).

4.1.2 Major phenolic constituents

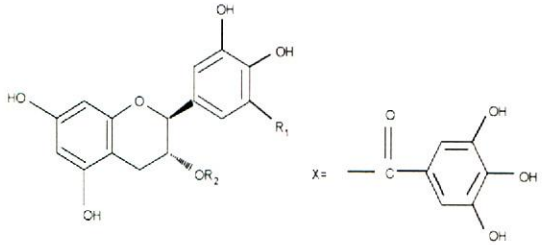
4.1.2.1 Tea flavonoids

The major polyphenolic fraction in green tea is the group of flavonoids made up of flavanols, i.e. the catechins (215,220). In addition to caffeine, the phenolic acid, gallic acid and the purines, theobromine and theophylline are also present as minor constituents. Partially "fermented" teas (oolong and paochong) contain both green tea catechins and black tea theaflavins (221). The principal catechins (Fig. 15) in fresh tea leaves and green tea are (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), (-)-epicatechin (EC), (+)-gallocatechin (GC) and (+)-catechin (C).



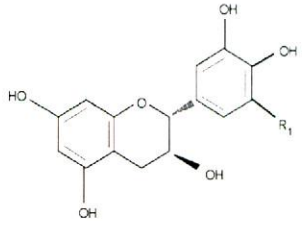
(-)-Epi-form (2R,3R)

(-)-Epicatechin: R1=R2=H
(-)-Epigallocatechin: R1=OH, R2=H
(-)-Epicatechin gallate: R1=H, R2=X
(-)-Epigallocatechin gallate: R1=OH, R2=X



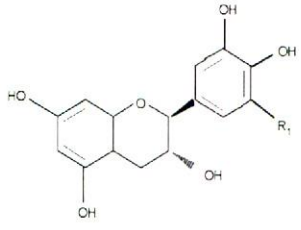
(-)-Form (2S,3R)

(-)-Catechin: R1=R2=H
(-)-Gallocatechin: R1=OH, R2=H
(-)-Catechin gallate: R1=H, R2=X
(-)-Gallocatechin gallate: R1=OH, R2=X



(+)-Form (2R,3S)

(+)-Catechin: R1=H
(+)-Gallocatechin: R1=OH



(+)-Epi-Form (2S,3S)

(+)-Epicatechin

Fig. 15. Major green tea catechins (222).

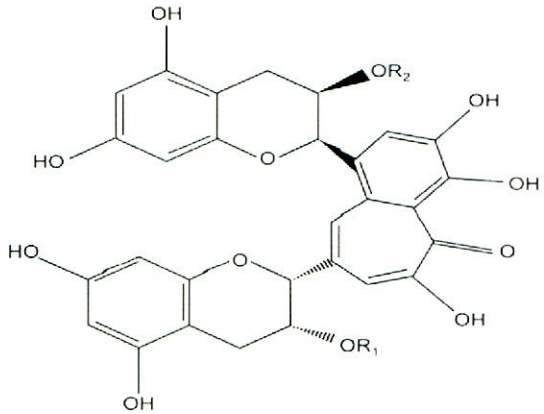
EGCG is the most abundant catechin accounting for 6-16% on a dry weight basis (223). EC contains an *ortho*-dihydroxyl group in the B-ring at carbons 3' and 4' and a hydroxyl group at carbon 3 on the C ring while ECG has a gallate moiety esterified at carbon 3 of the C ring. EGC has a trihydroxyl group at carbons 3', 4', and 5' on the B

ring while EGCG has an added gallate moiety esterified at carbon 3 on the C ring (207,214,215,220). Catechins are colourless, water-soluble compounds lending bitterness and astringency to green tea infusions. The taste, colour and aroma of manufactured tea are associated with modifications to the catechins (224).

The main flavonols in tea leaves are quercetin, kaempferol and myricetin and they make up 2-3% of the water-soluble extract (214). Flavonols are predominantly present as glycosides with the sugar moieties consisting of glucose, rhamnose, galactose, arabinose and fructose and mono-, di- and tri-glycosides. Variable values for flavonol glycosides content in teas are reported which could be ascribed to different sources of tea and varying processing procedures (225).

In the preparation of black teas, catechins are oxidised yielding the typical colour and flavour. The pigments of black tea have been divided into orange-red coloured theaflavins and brownish thearubigins (Fig. 16). Theaflavins are formed from polymerization of catechins during fermentation (226). There are four main theaflavins, theaflavin, theaflavin 3-gallate, theaflavin 3'-gallate and theaflavin 3,3'-digallate. The thearubigins are a heterogeneous group of phenolic pigments with relative higher molecular mass and are the major contributor to the black tea polyphenols (227). Theaflavins contain benzotropolone rings with dihydroxy or trihydroxy substitutions, with the thearubigins even more extensively oxidised and polymerised as illustrated in figure 17. A typically brewed black tea beverage contains approximately 3-10% (w/w) catechins, 2-6% theaflavins and >20% (w/w) thearubigins of the tea soluble solids per dry weight (214,215,228).

Quantitative and qualitative analyses of white tea identified nine of the major constituents found in green tea. EGCG was present at equally high levels in white tea when compared with levels in green tea. Higher levels of gallic acid, theobromine, ECG and caffeine were also found in white tea (219).



		R1	R2
Theaflavin	TF	H	H
Theaflavin 3-gallate	TF3G	Gallate	H
Theaflavin 3'-gallate	TF3'G	H	Gallate
Theaflavin 3.3'-gallate	TFDG	Gallate	Gallate

Fig. 16. Major black tea theaflavins (222).

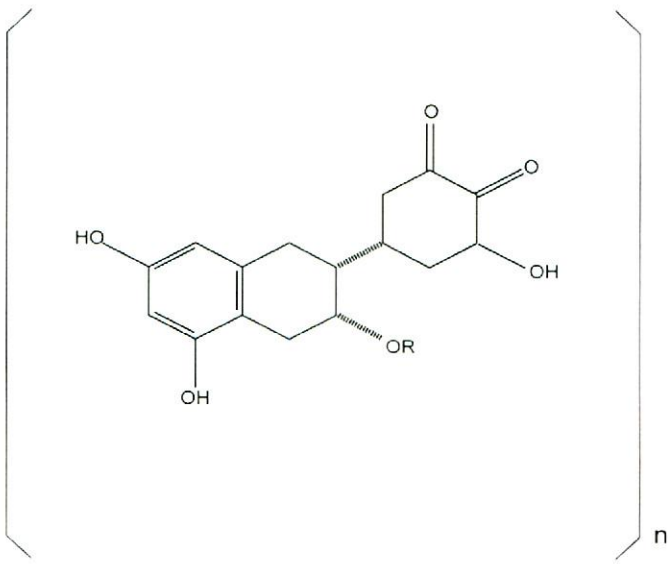


Fig. 17. Generic structure of thearubigins in black tea (215).

4.1.3 Metabolism of tea polyphenols

The metabolism of green tea catechins has been studied in various animals and humans showing that orally administered catechins are absorbed, metabolized and rapidly excreted within 24 hr in humans and 6-10 hr in experimental rats (229,330). Studies conducted in rats indicated that EGCG is mainly excreted through bile, while EGC and EC are excreted mainly through urine and bile (207,229). These studies are in agreement with another carried out by Chen *et al.* (231) where EGCG was absent from human urine samples, while EGC and EC was recovered. Metabolism and bioavailability of tea constituents are dependent on several factors including their structure, purity, dosage and route of administration. When green tea extract was consumed by healthy individuals, EGCG, EGC and EC were found in the plasma in concentrations varying from 0.2% to 2% of the ingested amount within 1.4 to 2.4 hr after ingestion (207). Saliva was found to have catechin esterase activity, suggesting that EGCG may be degalloylated in the mouth and oesophagus (230). A study conducted in rats suggested that catechins are either glucuronidated in the intestinal mucosa, sulphated in the liver and/or methylated in the liver and kidney. The major metabolites detected were the glucuronidated catechins (232). Human plasma also contains *O*-methylated and glucuronidated catechin metabolites and the authors suggested that the profile of conjugated metabolites may change at higher doses due to the saturation of specific metabolic pathways (232,233). EGCG is also absorbed by the skin when applied topically in a hydrophilic ointment (234). Information regarding the bioavailability and tissue levels of the tea constituents should feature prominently in future mechanistic studies, as only activities displayed by tea constituents at concentrations achievable in human tissues are of importance.

4.1.4 Biological activity of tea polyphenols

Due to the unique structures and activities of tea polyphenols they have a broad utility for treatment of various diseases or modulate many risk factors associated with common medical ailments. These include cardiovascular disease, cancer, oral health, bone health, thermogenesis, cognitive function, iron status, kidney stones, diabetes, immune responses, antibacterial activity, antiviral activity, dermatological therapy and various other (8,235). Many of these benefits are ascribed to the antioxidant properties and free radical scavenging ability of green and black teas. The catechins interact with numerous structurally unrelated molecules and may alter cellular membranes, proteins,

lipids, nucleic acids as well as cellular factors important for normal and abnormal cell growth which are the key to cancer development and metastasis. Some of these interactions could be responsible for the anticarcinogenic and antimutagenic properties of green and black teas (236,237). Despite all these findings, the understanding of the mechanisms involved in the biological effects of green and black teas are far from complete. However, these mechanisms are important to design strategies for prevention and treatment of cancer.

4.1.4.1 Antioxidant activity

***(i) In vitro* studies.** A number of studies, using different assays, have reported on the *in vitro* antioxidant activity of green, black and oolong teas as well as their respective phenolic constituents (238). Each assay differs in its measurement of antioxidant activity depending on the nature of the radical generating system and assay conditions applied. Some of these assays include (i) the FRAP (ferric reducing ability of plasma) assay, based on the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}), (ii) the TEAC (Trolox equivalent antioxidant capacity) assay based on the formation and scavenging of the $ABTS^{\cdot+}$ [2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation, (iii) the TRAP (total radical-trapping parameter) assay measuring the total peroxyl radical trapping capacity, (iv) the ORAC (oxygen radical absorbance capacity) assay measuring the decrease in fluorescence of phycoerythrin in the presence of the radical generator, AAPH [2,2'-azobis(2-amidinopropane) dihydrochloride] that generates aqueous peroxyl radicals at a constant rate and (v) the DPPH radical assay measuring the ability to donate a hydrogen atom to the stable free radical, 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) (239). Using the ORAC assay, green and black teas displayed a much higher antioxidant activity against peroxyl radicals when compared to garlic, kale, spinach and Brussels sprouts (240). In the FRAP assay green tea is more potent than black tea (241). A study done by Vinson and Dabbagh (242) showed that phenolic constituents of black and green teas are responsible for its antioxidant activity and that the pure catechins and phenolic acids were found to be more powerful than vitamins C, E and β-carotene in an *in vitro* lipoprotein oxidation model. The antioxidant activity of the catechins using the Rancimat method varies in the order EGCG > EGC > ECG > EC with EGCG being the single strongest antioxidant as well as the major catechin component of green tea (243,244). The linkage of gallic acid to the epicatechin or epigallocatechin structure via esterification at the 3 position increases the antioxidant

potential (146). The antioxidant effectiveness of purified tea components inversely correlated (r = -0.999) with the % polyphenol content which was not the case for aqueous extracts prepared from the various green, black and oolong teas. Tea flavonoids have also been shown to directly quench reactive oxygen species *in vitro* as well as chelate metal ions to prevent their participation in Fenton reactions (246). The ability of a compound to scavenge free radicals is partly ascribed to its reduction potential (hydrogen or electron donation). EGCG and EGC have a lower reduction potential than vitamin E, suggesting they are superior electron donors (246). The *in vitro* antioxidant capacity of the various teas and their constituents is dependent on the type of assay used and does not reflect factors such as bioavailability and metabolism. The fact that catechins are metabolised could play an important role in the *in vivo* antioxidant activity.

(ii) ***In vivo*** **studies.** Most animal studies utilise the thiobarbituric acid reacting substances (TBARS) assay to assess the effect of tea consumption on lipid peroxidation *in vivo*. Plasma TBARS were decreased in hamsters fed a diet containing 1% tea catechins, and Wistar rats consuming black tea (243,247). New Zealand white rabbits did not show any difference in the plasma TBARS after consuming green or black tea in their drinking water (248). A study conducted by Kim et al. (249) indicated that topical application of EGCG to guinea pigs protected against UV-B light induced oxidative damage by reducing the TBARS in the skin. Serafini et al. (250) were one of the very first groups to show an *in vivo* effect that both green and black teas increased the plasma antioxidant activity in humans. Since then, numerous other studies have been conducted to demonstrate that a single dose of tea enhances the plasma antioxidant capacity of healthy adults. Using the FRAP assay, a significant increase in the plasma antioxidant capacity was detected in humans consuming three cups of green or black tea, while in another study, subjects consuming 300 mL of green tea showed a significant increase in their plasma antioxidant activity, using the ABTS assay (251-254). Biomarkers of oxidative damage such as oxidized deoxynucleosides (8-hydroxydeoxyguanosine), thiobarbituric reactive substances (TBARS) or malondialdehyde (MDA), micronuclei formation, sister chromatid exchange and low-density lipoprotein (LDL) are often utilised as surrogate markers for *in vivo* antioxidant activity (215). In studies examining the effect of tea consumption on some of these biomarkers in humans, one study indicated a significant 22% decrease in plasma

TBARS after the consumption of approximately 10 cups/day of green tea for 4 weeks, while another study showed no significant changes in volunteers consuming 2-3 cups/day for 7 days (255,256). However, in a study conducted in 1999 where healthy volunteers consumed six cups of black tea per day, their plasma was not protected from lipid peroxidation ex *vivo* (257). The discrepancy between the powerful *in vitro* antioxidant efficacy and the lack thereof *in vivo* could be ascribed to the insufficient bioavailability of tea polyphenols in humans.

4.1.4.2 Prooxidant activity

Although plant polyphenols are good antioxidants that act as radical scavengers protecting the cell against oxidative damage, polyphenols also exhibit prooxidant activity under certain conditions, generating hydroxyl radicals especially in the presence of Fe (II or III), Ag (I) or Cu (II) [258,259]. Purified tea polyphenols, EGCG and ECG induce apoptosis in various cancer cells which is associated with the generation of H_2O_2. Yang *et al.* suggested that EGCG may serve as a prooxidant, but the relevance of this mechanism still needs to be studied *in vivo* (260) as transition metals found *in vivo* and in plasma are complexed by proteins and are unlikely to catalyse the autoxidation of polyphenols significantly (261).

4.1.4.3 Metal ion chelation

Dietary iron is available in two valence states, ferrous (Fe^{2+}) and ferric (Fe^{3+}) and the majority of Fe^{2+} is found in haem iron whereas the majority of Fe^{3+} is in non-haem iron. Tea consumption mainly influences the absorption of non-heme iron and the reduction of non-heme iron absorption in the presence of tea flavonoids suggests that chelation of iron are one of the mechanisms of antioxidant action *in vivo* (262,263).

The iron chelating activity of tea flavonoids may affect the function of various heme- or metalloproteins that are dependent on metal ions for activity (264). Tea polyphenols chelate metal ions such as iron and copper thus preventing their participation in free radical generating reactions (146,245,265). Iron and copper play a major role in the production of the very reactive hydroxyl radical ($HO^{\bullet}$) through the Fenton and Haber-Weiss reactions (266) and chelation of these metals will decrease the oxygen toxicity to cells. Green and black tea polyphenols bind ferric iron (Fe^{3+}) to form a redox-inactive Fe-polyphenol complex (265). The gallate esters, EGCG and

ECG, have greater chelating activity than EGC and EC and are active *in vivo* as consumption of green and black tea lowers absorption of dietary iron (266). Glycosylation at any of the functional hydroxyl positions would decrease the metal chelating activity of that particular flavonoid (175.)

4.1.4.4 Antimutagenic activity

In the mid 1980s Japanese and Chinese investigators reported that water extracts of green tea exhibited antimutagenic effects in bacterial test systems (267,268). Since then many *in vivo* studies in rodents were conducted with water extracts or phenolic fractions of green and black tea to investigate the possible protective effects of tea against cancer development. These studies show that tea protects against many types of cancers in skin, lung, oesophagus, stomach, liver, small intestine, pancreas, colon, bladder, prostate and mammary glands (269-276).

(i) *Salmonella* mutagenicity studies. *In vitro* studies using the *Salmonella* mutagenicity assay indicated that tea and tea components display antimutagenic activity against the major classes of dietary, environmental and occupational mutagens and/or carcinogens. These include heterocyclic amines, polycyclic aromatic hydrocarbons and their ultimate carcinogens, nitrosamines and other aromatic amines, pyridoindols and pyridoimidazols as well as UV light (55,277-281). Comparison of the antimutagenic activities of aqueous extracts of green, black and decaffeinated black tea conducted at concentrations customarily used for human consumption showed no major differences in the antimutagenic potential against three different metabolic activated mutagens (282). In a study conducted by Weisburger *et al.* (278) similar protective effects were observed for green and black tea polyphenols against groups of carcinogens or mutagens representing aromatic and heterocyclic amines, polycyclic aromatic hydrocarbons and N-nitrosamines, with no effect on the mutagenicity of 1-nitropyrene and 2-chloro-4-methylthiobutanoic acid. Apostolides *et al.* (283) showed that purified black tea polyphenols have slightly better protective effects against PhIP-induced mutagenicity than green tea polyphenols.

Relatively fewer reports are found on studies comparing black tea with green tea with regards to antimutagenicity (284). A common finding though was that the inhibition of mutagenesis is associated with the polyphenol fraction of both green and black teas.

Many studies attempt to identify the active component(s) responsible for green and black tea's good antimutagenic and/or anticarcinogenic activities (280,285). These studies suggest that different degrees of tea fermentation might produce different types of antimutagenic compounds effective against different mutagens (286).

It is apparent from the available data that the cancer chemopreventive activity of green and black tea is a combined effect of several active components, as shown by Suganama *et al.* (287) where the action of EGCG in the presence of EC and that of a whole green tea infusion showed better protective activity than EGCG alone in lung cancer cells.

Recently a study conducted by Santana-Rios *et al.* (219) reported on the potent antimutagenic activity of white tea against a number of heterocyclic amines compared with green tea in the *Salmonella* assay. The greater inhibitory effect exhibited by the white tea was proposed to be related to higher concentrations of several of the major phenolic constituents and a possible synergistic effect with the minor constituents to prevent mutagenicity, presumably via multiple mechanisms.

(ii) Animal studies. Tea and its constituents inhibit chemically-induced carcinogenesis in various animal models. Yoshizawa *et al.* (288) were the first to use the two stage skin carcinogenesis mouse model to demonstrate that the topical application of the green tea flavonoid, EGCG inhibited tumour promotion in DMBA-initiated, teleocidin-promoted mouse skin while Katiyar *et al.* (62) showed an inhibition of TPA promotion utilizing the same mouse skin model. Since then many studies demonstrated that topical application or oral feeding of phenolic extracts and individual catechin derivatives of green and black tea has anticarcinogenic activities in animal skin studies (274,289-291). To elucidate the mechanisms involved, various studies showed that the tea inhibits DNA synthesis and enhances apoptosis. A study conducted by Saha and Das (292) indicated the activation of several detoxification enzymes, decreased cellular damage, inhibition of cell proliferation and induction of apoptosis in the liver of DMBA-initiated, croton oil-promoted mice consuming aqueous black tea, black tea polyphenols and EGCG. Topical application of black tea polyphenols also inhibits markers of inflammatory responses such as epidermal edema, hyperplasia, leukocyte infiltration, induced activity of epidermal ornithine decarboxylase and cyclooxygenase (207).

In addition to preventive effects against skin carcinogenesis, studies indicated that green and black tea polyphenols are also effective against chemically induced carcinogenesis of various internal organs. Oral consumption of green or black tea water or phenolic extracts demonstrated a protective effect against N-nitrosodiethylamine and benzo[a]pyrene-induced forestomach and lung tumourigenesis in A/J mice (274,293). When the tobacco smoke carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was used to induce lung tumorigenesis in mice and rats, green, black tea extracts and EGCG and theaflavins afforded effective protection (153,276,293). Tea consumption and digestive tract cancers have also been studied and recently Morse *et al.* (294) reported that theaflavin significantly reduced NMBA-induced oesophageal tumour formation, while other studies showed high doses of theaflavin, black or green tea polyphenols or EGCG reduced the tumour multiplicity but not the oesophageal cancer incidence when orally administered to rats (272,295). Oral feeding of EGCG also resulted in a reduction of N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG)-induced duodenum tumourigenicity in C57BL/6 mice (296). Different studies also reported on the effects of black and green tea on azoxymethane (AOM)-induced colon cancer in rats, with one study reporting on the inhibition of AOM-induced carcinogenesis following oral feeding of green tea polyphenols in drinking water of Fischer rats (297). The oral feeding of a tea infusion with or without the addition of milk during the period of carcinogen administration significantly decreased the production of aberrant colon crypts (298). When heterocyclic amines obtained from grilled and/or cooked meat, were used in animal studies green tea, EGCG and black tea polyphenol fractions also inhibited the formation of aberrant crypts in the colon with a synergistic effect between green tea and phytic acid (299,300). Beneficial effects of tea have also been observed in rats, mice and hamsters in the inhibition of liver carcinogenesis. The administration of green tea leaf in the diet of rats prior to and after DEN and AFB_1 treatment resulted in a significant inhibition of χ-glutamyl transpeptidase-positive foci in the liver (272,301). Tea catechins, black tea and oolong tea extracts significantly decreased the number and area of preneoplastic GST-P positive foci in the liver of DEN-initiated, PB-promoted liver carcinogenesis (302). Recently, the protective effect of tea on the development of mammary gland and prostate cancer has also been investigated. Black and green catechins had no significant inhibitory effect on 7,12-diemethylbenz[a]anthracene mammary gland carcinogenesis in rats, while EGCG, administered via intra peritoneal route, inhibited the growth and reduced the size of tumours initiated by human prostate and breast cancer cell lines inoculated into mice and rats (303,304). Results from these

studies indicated that tea is active against carcinogenesis in animals for skin, lung, colon, liver, oesophagus, forestomach and hormonal-dependant cancers.

(iii) Studies in humans. Epidemiological studies on the relation between tea consumption and altered cancer risk have been inconsistent. This could be ascribed to the lack of proper study design as most of the studies were not specifically designed to assess the effect of tea consumption on minimising the risk factors for cancer development. Differences in lifestyle habits, type of tea and duration of tea consumption in the different study populations and potential confounding factors could also be contributors to the inconsistent findings (180,305,306). One Japanese cohort study found a significant inverse association between total cancer risk and green tea consumption in women who consumed more than 10 cups per day (307). In a study designed to determine the tea intake patterns in an older south western United States population, a significantly lower risk of squamous cell carcinoma (SCC) of the skin was associated with hot black tea consumption (306). The consumption of black tea was shown to reduce the risk of rectal cancer in women and men in a Russian population based case-control study (308). In China, a case control study indicated an inverse association with ovarian cancer, concluding that an increased frequency and duration of green tea consumption may reduce the risk of ovarian cancer, while further investigations are required for black and oolong teas (309). A more recent nested case-control study conducted in Japan to evaluate whether green tea consumption provides protection against development of stomach cancer also showed no inverse association, which is in accordance with four other prospective studies (310). These results confirm the need for future studies with the goal of examining green and black tea intake in relation to the reduction of cancer risk in humans in much more detail.

4.1.5 Proposed protective mechanisms

Anticarcinogenic compounds have been classified into three categories according to their proposed mechanisms of action i) compounds inhibiting the formation of mutagenic and/or genotoxic metabolites from precursor molecules, ii) "blocking" compounds inhibiting the reactions of carcinogens or scavenging radicals and iii) "suppressing" compounds which inhibit the expression of the malignant characteristics at cellular level (311,312). Multiple mechanisms seem to exist whereby tea and its phenolic constituents elicit their inhibitory effects against mutagenesis and/or carcinogenesis and these

include inhibition of the metabolic activation of carcinogens, increased detoxification via the induction of phase II enzymes; inhibition of oxidative damage to DNA and proteins, inhibition of enzymes involved in the formation of reactive oxygen species and radicals and influencing cellular and molecular events (313). Other mechanisms relating to antiviral activity and enhancement of immune functions have also been suggested but their relevance to carcinogenesis still needs to be elucidated.

4.1.5.1 Modulation of drug metabolising enzymes and oxidative stress

Both phase I and phase II enzymes are induced by rats consuming green or black tea extracts (314). A variety of green tea polyphenols have been shown to alter rat liver microsomal mono-oxygenase activities. Exposure of rats to sub-chronic doses of green and black teas caused an induced activity of specific classes of P450, e.g. 1A1, 1A2, and 2B1 with no effect on the other phase I enzymes (300,314,315). Green tea catechins inhibited aryl hydrocarbon hydroxylase (AHH) activity in liver and in epidermal microsomes as well as tissue enzyme-mediated binding of tritiated B[a]P to DNA. These results all suggest that the antimutagenic potential of green tea polyphenols may be in part, related to their ability to inhibit P450-dependent metabolic activation of PAHs, which would result in the inhibition of PAH-DNA adduct formation (237). The inhibition of P450-dependent bio-activation may be due to impairment of the electron flow from NADPH (222).

The enhanced generation of reactive carcinogen metabolites is not necessarily to a cell's disadvantage, as tea polyphenols also increase the detoxification rate by simultaneous induction of phase II detoxifying enzymes. Chronic feeding of mice with green tea extracts in their drinking water significantly enhanced the activities of phase II enzymes such as glutathione-S-transferase, glutathione peroxidase, catalase and NADPH-quinone oxidoreductase in the bowel, lung and liver (316), while Bu-Abbas and co-workers showed stimulation of hepatic UDP-glucuronosyl transferase activity (317). A study conducted by Sohn *et al.* (314) indicated a significant increase in the activity of UDP-GT as well as increased levels of GSH in the plasma of male Fischer rats consuming aqueous extracts of green and black teas. Oral feeding of green tea leaves in the diet of male Wistar rats resulted in significant increases in the activities of the antioxidant enzyme, catalase and the phase II detoxifying enzyme, GST-α. Also noted were elevated levels of GSH in the liver (318). Various *in vitro* studies have confirmed

the protective effect green tea polyphenols have against oxidative injury by minimising the generation of ROS, decreasing lipid peroxidation and stabilising the endogenous antioxidant GSH, thus enhancing the ability of the cells to cope with oxidative stress. (319-320).

Other mechanisms hypothesized include induction of DNA repair and binding with activated carcinogens. The antimutagenic and/or anticarcinogenic effect of teas may be partly due to changes in hepatic drug metabolizing enzymes (222).

4.1.5.2 Cell cycle regulatory effects

Many studies indicate an association between cell cycle regulation and cancer via the modulation of cell proliferation and apoptosis (322). In eukaryotes regulation of the cell cycle is partly controlled by a family of protein kinases complexes. These complexes consist of a catalytic unit, the cyclin-dependent kinase and an activating unit, the cyclin, which are activated at specific intervals during the cell cycle. Exogenous factors have been suggested to induce and regulate these complexes (322). EGCG irreversibly causes G0/G1-phase arrest of the cell cycle in A431, a human epidermoid carcinoma cell line (322). EGCG and EGC also inhibit the proliferation of various cancer cell lines as measured by [^{3}H]thymidine incorporation, with some cells requiring higher concentrations while other are more sensitive to lower concentrations. EGCG is the most active inhibitor of proliferation of certain breast cancer, colon cancer and melanoma cancer cell lines, whereas EGC is more active against a lung carcinoma cell line (323). Fujiki *et al.* (324) reported that tea polyphenols cause a G2/M cell cycle arrest and growth inhibition in a human lung cancer cell line. Green and black tea polyphenols also strongly inhibit DNA synthesis in DS19 mouse erythroleukemia cells and cell growth in four human tumour cell lines (323,325).

The inhibitory activities of tea and tea polyphenols may also be due to their ability to inhibit growth-related signal transduction pathways. AP-1 (activator protein), an oncogene, is involved in cell transformation and progression of cancer, and NFκB (nuclear factor kappa B) is an anti-apoptotic factor which promotes survival in cancer cells. The inhibition of AP-1 dependent transcriptional activity occurs via the inhibition of a *c-jun* NH_2-terminal kinase-dependent pathway. EGCG, EGC and theaflavin-3,3'-digallate have been shown to inhibit AP-1 activation and the phosphorylation of *c-jun* and an extracellular signal-related protein kinase (Erk). Inhibition of the transcription

factors, NF-κB and AP-1 as well as the reduced activity of protein tyrosine kinase and *c-jun* mRNA expression by EGCG may also contribute to the anticarcinogenic effect of the tea and its polyphenols (224,326). Recently, another mechanism by which EGCG induced apoptosis was reported, suggesting that EGCG specifically binds to Fas, also known as APO-1 or CD95, presumably on the cell surface, triggering the cascade of Fas-mediated apoptosis in U937 cells (327).

Cell cycle inhibitors are viewed as being the new generation of anticancer drugs and some synthetic compounds are already being investigated in clinical trials. Natural occurring agents like tea polyphenols could therefore play an important role as potential chemopreventive agents (322).

4.1.5.3 Anti-inflammatory activity

An association between chronic inflammation and increased cancer risk has been demonstrated by various epidemiological studies (328). Phagocytes secrete chemically reactive oxidants, radicals and electrophillic compounds during the process of inflammation which can damage surrounding tissue leading to DNA adduct formation and somatic mutations resulting in malignant transformation. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are considered as major bio-determinants in the process of cancer development (328,329). In this regard, the formation of nitric oxide (NO) is increased during infection and inflammation, possibly promoting carcinogenesis.

Tea polyphenols react with nitrosating species, inhibiting nitrosation (330,331). Humans consuming green and black tea also demonstrated the inhibition of N-nitrosoproline formation and it was estimated that the consumption of 3-5 g of tea per day may effectively block nitrosation reactions in humans (332). Green tea polyphenols also inhibit both *in vitro* and *in vivo* nitrosation reactions (333) which are regarded as a major source for the endogenous formation of secondary and tertiary amines in humans. There exists a two-edged situation similar to the antioxidant and/or prooxidant activities of tea, as tea polyphenols at low concentrations can also be nitrosated to form C-nitroso derivatives which can act as potential catalysts of other nitrosation reactions (272).

The green tea catechin, EGCG was shown to block the induction of nitric oxide synthase (iNOS) by down-regulating the activity of transcription factor NFκB (nuclear factor kappa B) in macrophages (326). Another study by Paquay *et al.* (334) confirmed the protective effects of green and black tea against NO toxicity in a pulmonary macrophage cell line. The tea and tea components not only scavenged NO and peroxynitrite, but also inhibited the activity as well as the induction of iNOS. In mouse skin the induction of inflammation is mediated by chronic topical application of the phorbol ester tumour promoter, TPA. Topical application of black tea polyphenols significantly inhibited the markers of inflammation, e.g. induction of epidermal ornithine decarboxylase (ODC), cytokine IL-1α mRNA expression and cyclooxygenase activity (335).

4.1.6 Tea tannins and human health

Hurrel and co-workers showed that black tea, when consumed simultaneously, inhibits the bioavailability of non-heme iron by 79 to 94% (336). Consumption of green tea extracts with a meal also reduces the non-heme iron absorption by 25% (263). Iron deficient anaemia among children in Saudi Arabia and the United Kingdom, may be aggravated by the regular consumption of tea with meals (337,338). Tea is not likely to cause iron deficiencies in healthy individual consuming a well balanced diet that includes readily available sources of heme-iron. Vegetarians and other people at risk for iron deficiency anaemia, e.g. infants, young children and pregnant women, should be encouraged to drink tea between meals and with added milk or lemon juice, limiting the chelating activity (339,340).

Obtaining significant effects through diet supplementation with polyphenol extracts seem to be very difficult without decreasing the nutritional value of diets, especially the reduction in digestibility and protein efficiency. This was shown in a study conducted by Zdunczyk *et al.* (341) where the addition of large amounts of catechin extracts (0.8%) effectively reduced the total cholesterol and LDL fraction in the serum, but also caused a reduction in protein digestibility and protein efficiency. It was mentioned that catechin extracts from green tea leaves and flavones from skullcap exhibited more favourable biological properties compared to anthocyanins and condensed tannins.

4.1.7 Caffeine and human health

Caffeine (1,3,7-trimethylxanthine), a natural alkaloid is probably the most frequently ingested pharmacological substance in the world, as it is found in coffee, tea, soft drinks, cocoa products and certain medications. Tea contains between 20-73 mg/100 mL (342) and variation of the content is dependant on preparation as well as the type of tea. Upon ingestion, caffeine is absorbed from the gastrointestinal tract into the bloodstream and can also pass the blood-brain barrier and placenta (343). Caffeine is primarily metabolised in the liver. Infants up to the age of 8-9 months have a reduced ability to metabolise this alkaloid (344). People differ in sensitivity to caffeine and need to be aware of possible adverse effects such as altered calcium balance in bones, increased anxiety, increased time to fall asleep, reduced fertility and interference with foetal growth (345). In growing children it is unknown if long-term daily consumption of caffeine would produce effects such as hyperactivity and anxiety as no study has been designed to test for potential chronic effects. The nervous system, including the brain, continues to develop and mature throughout childhood and it is possible that the delayed development of the nervous system may render children more sensitive to any adverse effects of caffeine. In healthy adults, the moderate consumption of caffeine has not been associated with any major adverse effects. Based on data from many studies, a dose level of 6 mg per kg bodyweight per day caffeine for healthy adults was established without adverse effects and children should not exceed an intake of 2.5 mg caffeine per kg bodyweight per day (346).

4.2. *Aspalathus linearis* (Rooibos tea)

4.2.1 History and processing

Rooibos (pronounced "roy boss") tea has been used as herbal beverage and to a lesser extent, as herbal medicine, in South Africa since the 1800's (347). The indigenous Khoi people were the first to use rooibos tea as a herbal beverage and in 1904 a Russian immigrant, Benjamin Ginsberg, began trading it from the San tribe. Just before the Second World War, his son, Charles Ginsberg started what is considered today as the "heritage" rooibos brand, Eleven O'Clock Rooibosch Tea. It was in the 1930's when a local medical doctor and nature lover, Dr P.F. Le Fras Nortier, realised its commercial potential and initiated cultivations in plantations by various local farming communities (http://www.rooibosltd.co.za/storyof.htm). Ceylon tea was in short supply during the

Second World War, while production levels of rooibos grew steadily. However, after the war the rooibos market collapsed and in an effort to rescue the industry the producers established the Clanwilliam Tea Cooperative in 1948. In 1954, the cooperative became the Tea Control Board with the goals being regulation of marketing, stabilization of prices and standardization of the quality. In 1993, the governmental Tea Control Board was privatised and the company Rooibos Ltd was established (http://www.rooibosltd.co.za/storyof.htm) owned largely by rooibos producers.

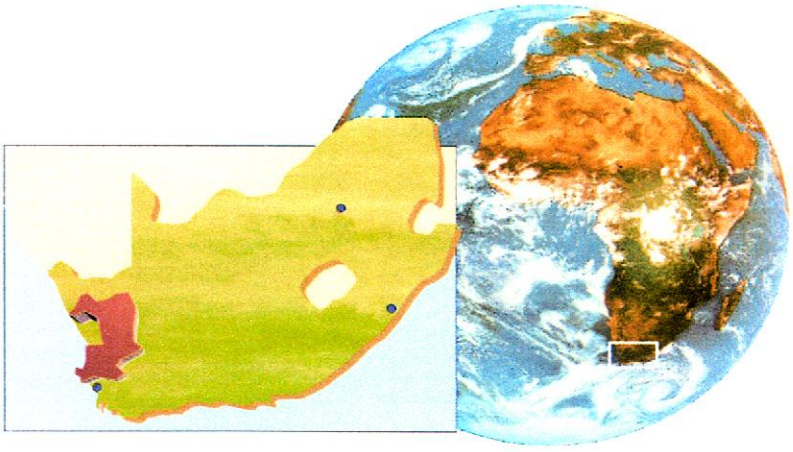


Fig. 18. – Map of Western Cape Province, South Africa with Clanwilliam region indicated in red (Rooibos Ltd, Clanwilliam, South Africa).

Today rooibos is cultivated on over 23 000 hectares of land and includes over 350 commercial farmers in the Clanwilliam region of the Western Cape Province (Fig. 18) and less than 200 small-scale farmers located in the town Wupperthal in the Cedarberg mountain area of the Western Cape Province and in Southern Bokkeveld, an area south of Nieuwoudtville in the Northern Cape (http://www.gem.org.za; http://www.wesgro.org.za). The popularity of this herbal tea, both locally and abroad can be ascribed to the low tannin content and absence of caffeine (348,349). The annual quantity rooibos tea produced in 2003 was ~9500 tons, with total sales for 2003 being 10500 ton of which 62% was exported (Personal communication, Arend Redelinghuys, Rooibos Ltd). The European market, mainly Germany and the Netherlands, accounts for the highest export volumes from South Africa, followed by Japan and the United Kingdom. About 30% of exported rooibos teas are flavoured varieties. There is also an increasing demand for organic and "green" or unprocessed rooibos tea and many farms are in the process of converting to organic production (http:www.emg.org.za/Documents/rooibos.pdf).

Aspalathus linearis (Fig. 19), belonging to the *Fabaceae* family (*Leguminosae*), is endemic to the Cedarberg region of the Western Cape, a mountainous area 250 km north of Cape Town. Only here can one find the unique combination of soil, altitude and climate necessary for the plant to grow.

Fig. 19. The rooibos plant, *Aspalathus linearis* (Courtesy of Rooibos Ltd, Clanwilliam, South Africa).

A closely related species, *A. pendula* Dahlg., as well as several wild types of *A. linearis* are used to prepare rooibos tea but on a non-commercial scale. Rooibos requires sandy soil with high acidity and sparse but consistent rainfall, is seeded between February and March and harvested 18-24 months later. The field can be harvested three more times, where after the land is ploughed and left for five years before it can be replanted. During this time the farmers fertilize the soil with other crops such as wheat or potatoes. The plant is a shrub-like bush with a central, smooth barked main stem and divides into a number of strong offshoots near the soil, followed by side branches bearing the needle-like leaves and stems that are used to manufacture rooibos tea (http://www.rooibosltd.co.za). The plant's height varies from 1 to 1.5 m in its natural state. Branches are cut either by hand with a sickle or by machine. Two types of rooibos tea can be produced depending on the processing methods. Manufacturing processed rooibos tea entails cutting the leaves and stems into uniform lengths of 3-5 mm, thoroughly wetting the cut leaves and stems before it is bruised between rollers to induce chemical oxidation or "fermentation" in heaps (Fig. 20).

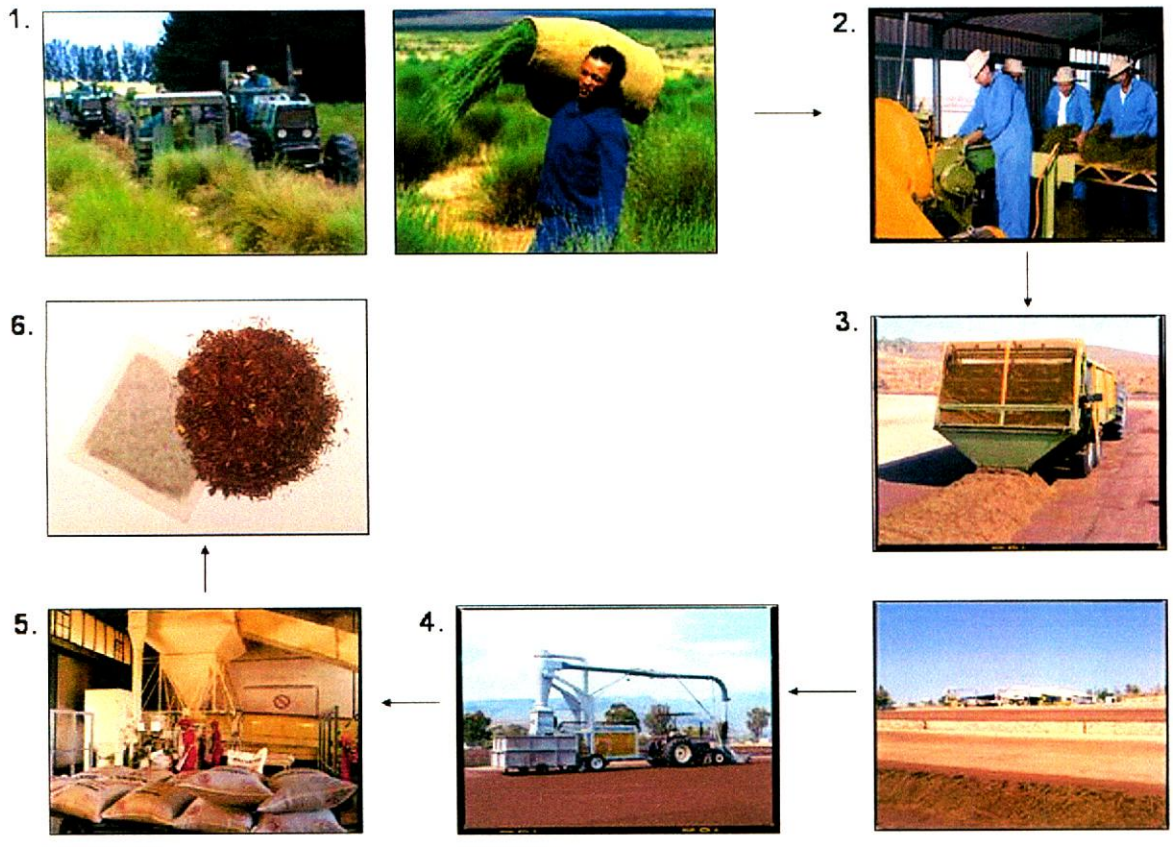


Fig. 20. Processing steps in the manufacturing of rooibos tea (Courtesy of Rooibos Ltd, Clanwilliam, South Africa).

During the oxidation step, the distinctive red colour, aroma and flavour of rooibos tea are developed. After the oxidation process, which lasts from 12-24 hrs depending on the temperature of the heap, the cuttings are thinly spread out in hot sunlight to dry. Further processing is then accomplished at the factory and includes sieving, grading, steam pasteurisation and drying over a hot air-bed dryer. Rooibos tea is subjected to strict microbiological tests before it is made commercially available. The rooibos is then weighed, packed, either in bags or in loose leaf form, and marketed under various brand names (personal communication, Arend Redelinghuys Rooibos Ltd).

Another type of herbal tea is the unprocessed/"unoxidised" or "green" form of rooibos tea which is manufactured by immediately drying the harvested plant material in an oven before cutting it into pieces and sieving it, preventing chemical oxidation to take place. During the oxidation step when preparing the processed rooibos tea, a substantial loss of the major flavonoid, aspalathin, occurs as well as a decrease in antioxidant activity (350,351). This warranted the development of a minimally oxidised

product, the "green" rooibos (353). Unprocessed rooibos tea has only recently come onto the South African and overseas markets.

4.2.2 Phenolic constituents

The phenolic constituents of rooibos tea (Fig. 21) differ from that of green and black teas, and are unique as rooibos tea contains aspalathin, only isolated from rooibos and nothofagin, another rare β-hydroxydihydrochalcone (353). The dihydrochalcone content of rooibos decreases substantially during processing, with less than 7% of the original aspalathin content remaining in the processed tea (354).

Apart from the aglycone, quercetin, other flavonols present in rooibos, is the quercetin glycosides, rutin and isoquercitrin (353). According to Toyoda *et al.* (355) rooibos contains the flavonol, kaempferol, but this identification is based only on HPLC analysis. The flavones comprise orientin, iso-orientin, vitexin, chrysoeriol, 5,7,4′-trihydroxy-3-methoxyflavone and luteolin (353). The flavanol, (+)-catechin (356) is present, which together with (-)-epicatechin, form the chain-extending units of the rooibos procyanidin type tannin (357). A cup of processed rooibos tea (150 ml) is estimated to contain 1.4 mg of aspalathin (354) and 1.5 mg quercetin (358). Recently a HPLC method, using a C_{18} reverse phase column with mass spectrometric analyses, was developed that allows for sensitive and reproducible quantitation of the main flavonoids in rooibos tea (359). With growing interest in the unprocessed rooibos tea, Schulz *et al.* (351) developed a near-infrared spectroscopy (NIRS) method to rapidly discriminate between processed and unprocessed rooibos tea while predicting the aspalathin content in the unprocessed form. The aspalathin content of the unprocessed rooibos samples was estimated at 35 to 68% of the total phenolic content.

4.2.3 Biological activities of rooibos

4.2.3.1 Anecdotal health claims

South African consumers and physicians use rooibos tea for a wide range of ailments, such as digestive disorders, skin allergies, loss of appetite, nervous tension, and insomnia. In 1968 a young South African mother, whose baby was allergic to milk and suffered with colic, fed her baby rooibos tea and was astonished at the results: the baby gained weight, and was cured of the chronic restlessness, vomiting and stomach cramps. She published all her observations in a book: Babas, Allergieë en Rooibostee,

(translated "Babies, Allergies and Rooibos tea") in 1974 and communicated the findings through the press and other public forums (348). The antispasmodic activity of the tea was later suggested to be ascribed to two components isolated from processed rooibos tea, quercetin and luteolin (358).

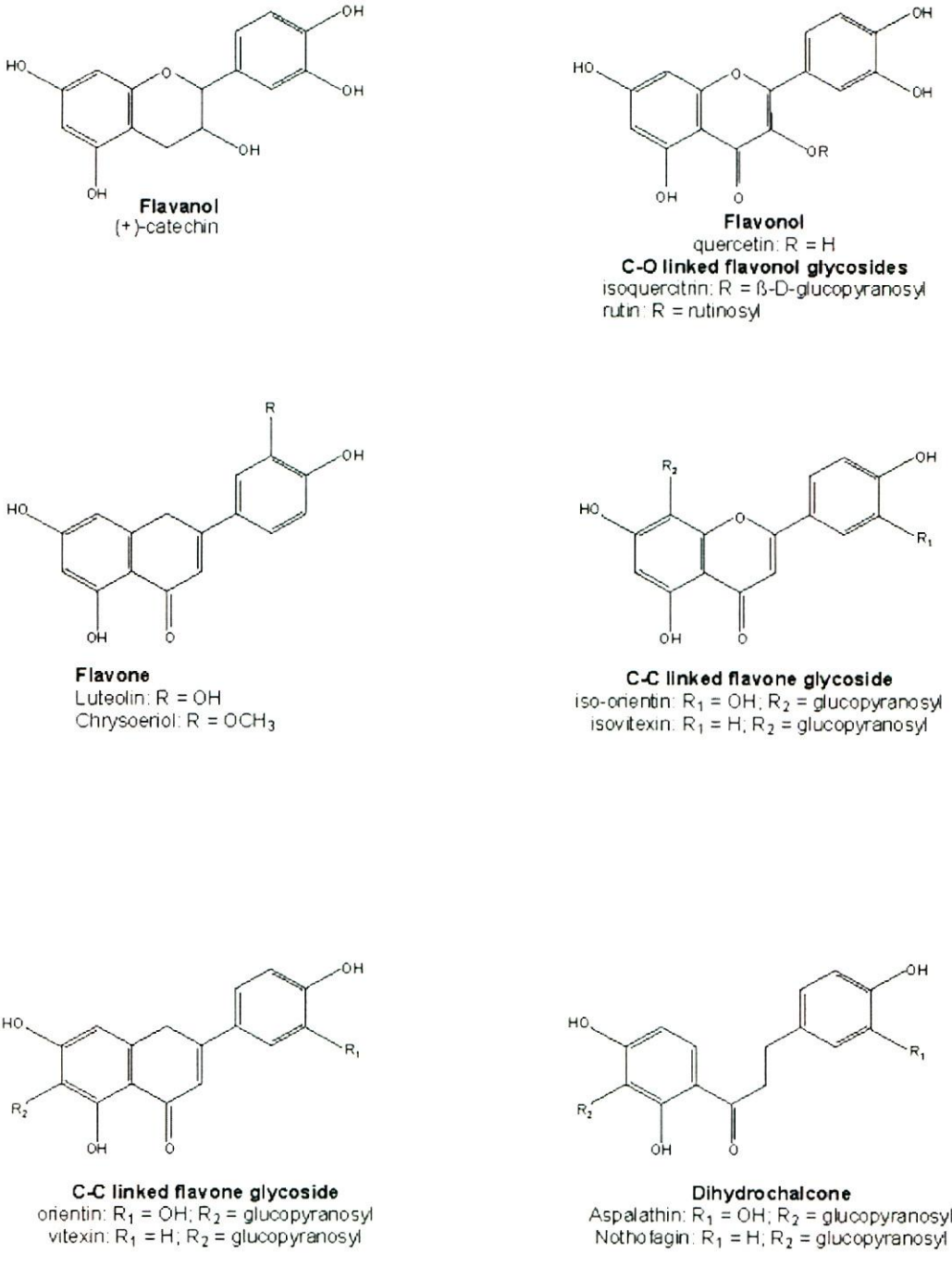


Fig. 21. Structure of the main polyphenolic constituents of rooibos tea (356).

4.2.3.2 Antioxidant activity

One of the earliest references to the antioxidant activity of rooibos tea was the study conducted by Yoshikawa *et al.* (360) who assessed the antioxidant activity of rooibos tea by measuring reactivity to various reactive oxygen species using electron spin resonance (ESR) spectrometry. Aqueous extracts of rooibos tea (processed form) effectively scavenged the superoxide anion radicals and hydroxyl radicals. Subsequently, Ito *et al.* (361) reported that aqueous extracts of rooibos tea (processed form) could protect mouse leukaemic cells against H_2O_2-induced oxidative stress. Rooibos tea extracts also inhibited the growth of the primary cells, myoblasts and fibroblasts, in tissue culture. The authors suggested that this inhibitory effect could be due to the potent scavenging activity of rooibos tea, but other compounds having cytostatic properties were not excluded (362).

The antioxidant properties of specific rooibos tea fractions, e.g. ethyl acetate soluble and insoluble fractions of an aqueous extract, were determined in a study by Von Gadow *et al.* (363). The ethyl acetate soluble solids were more effective antioxidants than the insoluble fraction containing polymeric solids on a mass basis. Extraction of the water soluble fraction with ethyl acetate removed the phenolic acids and flavonoids and these compounds have been shown to be potent antioxidants. Selective extraction is important when a maximum antioxidant potential of rooibos tea solids is required. The aspalathin content in commercial unprocessed rooibos tea samples was shown to correlate well (R^2=0.812) with the total antioxidant activity using the ABTS-scavenging method (351). Aspalathin showed a higher ability to scavenge the DPPH radical when compared with BHT and α-tocopherol, but offered less protection against lipid peroxidation (364). The hydrogen donating and superoxide radical scavenging abilities were also conducted with the ethyl acetate and crude aspalathin fractions being the most effective radical scavengers, followed by the aqueous extracts, crude polymeric fractions and tannin showing the weakest effects (365,366).

When comparing the antioxidant activity in three model lipid systems, sunflower oil-in-water emulsions, linoleic acid emulsion and methyl linoleate micelles, aqueous extracts of both processed and unprocessed rooibos tea, crude fractions and tannin possess antioxidant activity. The processed and unprocessed aqueous rooibos extracts were the most consistently effective in all the lipid systems used, suggesting these extracts are better equipped to handle oxidation in these various lipid systems (365).

The antioxidant activity of an aqueous extract of rooibos tea was determined using the Rancimat and β-carotene bleaching methods to assess the effect of extraction time and additional exposure to heat. The additional heat treatment and increased time during extraction increased the antioxidant activity (367).

Rooibos tea extracts contain less antioxidant activity than green, oolong and black teas on a cup for cup basis (363). As black and green teas have twice as much soluble solids as rooibos tea, the serving of rooibos tea to be consumed could be increased to obtain the same antioxidant benefit as measured by DPPH method (363). The results from the scavenging abilities correlated well with the total polyphenol content of the extracts and fractions. Tea processing reduces the antioxidant activity of rooibos tea (350).

4.2.3.3 Anti-HIV activity

Alkaline extracts (1% sodium carbonate) of processed rooibos tea suppress the HIV-induced cytopathic effect of HIV-1 infected MT-4 (Human T lymphotropic virus type 1 infected T cell line) cells *in vitro* (368). The polysaccharide isolated from the alkaline extract also exhibited very low cytotoxicity to the cells. The proposed mechanism(s) involved in the anti-HIV activity was the inhibition of the virus binding to the cells. Substances inhibiting the binding of the virions to the conserved region of the CD4 antigen are desired as it is active at a very early stage of the viral infection. The authors suggested that an extract like this should be a good candidate drug and will add new dimension to the therapeutic weapons against AIDS.

4.2.3.4 "Anti-ageing" properties

Aqueous extracts of processed rooibos tea were fed to Wistar female rats for 21 months and the damage to the central nervous system (CNS) assessed by thiobarbituric acid levels (TBA) and the level of lipidperoxides in the brain by magnetic resonance imaging (MRI). When compared with rats consuming water for 21 months, the TBA content was significantly lower in the rats consuming rooibos tea as well as in the young (5 weeks old) control group of rats. The MRI scans showed similar results for the rooibos tea treated rats and young rats when compared with the rats consuming water. Age-induced deterioration of the CNS is partly due to the cytotoxic effect of reactive oxygen species (ROS) generated in the brain. As rooibos tea exhibits potent

antioxidant activities by scavenging oxygen, hydroxyl and DPPH radicals (360,366), the results suggested that tea flavonoids suppressed the accumulation of lipid peroxides in the brain associated with the aging process (369).

4.2.3.5 Antibody production and cytokine generation

Impaired immune responses are known to cause allergies, along with other autoimmune diseases. A study was conducted to verify rooibos teas' control of allergic diseases, as many antioxidants inhibit histamine release from mast cells (370). Female Wistar rats and BALB /c mice were injected with ovalbumin (OVA) and fed aqueous extracts of rooibos tea. Cyclopsorin A was administered to the Wistar rats intravenously before OVA challenge while murine splenic B cells were cultured with various concentrations of aqueous rooibos tea extracts. Rooibos extracts increased the antibody responses and improved cell survival via the stimulation of IL-2. The authors suggested that phenolic compounds of rooibos tea were responsible for these biological activities, as removal thereof diminished the antibody production (370).

4.2.3.6 Antihemolytic properties

The antihemolytic activity of rooibos and black tea on Japanese quail erythrocytes was studied. Peroxide and hypotonic haemolysis of the red blood cells of quails, either fed with rooibos tea supplemented food or fed without tea, was performed. Long-term consumption of rooibos tea did not change the erythrocyte fragility to either peroxide or hypotonia induced haemolysis. However, rooibos and black teas decreased peroxide induced haemolysis of erythrocytes but not haemolysis induced by a hypotonic NaCl solution. An increased inhibition of haemolysis was been obtained when a boiled water extract of rooibos tea was used. The degree of inhibition was comparable with the effect of ascorbic acid (371).

4.2.3.7 Antimutagenic activities

The protective effect of rooibos tea against BP, mytomycin C and γ-rays were investigated. Aqueous extracts of processed rooibos tea administered (via drinking water or gastric intubations) to male ICR mice, subsequently treated with benzo[a]pyrene (BP), mytomycin C or γ-rays reduced the frequency of micronuclei in

peripheral blood reticulocytes. Chromosome aberrations were also suppressed in rooibos tea treated Chinese hamster ovary cells after exposure to BP and mytomycin C. The suppressing effects of rooibos tea were better when cells were treated with a combination of the extract and BP and mytomycin C, than when cells were treated separately (373,373). Komatsu *et al.* (374) also reported a protective effect of rooibos tea extract using x-ray-induced cell transformation of C3H10T1/2 mouse cells. The suppression was dependent on the duration of treatment. Green tea, at an equivalent concentration, displayed no detectable effect on the transformation incidence (374).

4.2.3.8 Hepatoprotective effect

The hepatoprotective effect of an aqueous extract of commercially obtained rooibos tea on cirrhosis development by carbon tetrachloride (CCl_4) intoxication in male Wistar rats was investigated (375). Reactive metabolites of CCl_4 cause lipid peroxidation, producing hepatocellular damage and enhancing the production of fibrotic tissue. Administration of rooibos tea protected the rats against liver damage, as indicated by the histological regression of steatosis and cirrhosis in the liver tissue as well as a significant inhibition of the increase of liver tissue concentration of malondialdehyde (MDA), triacylglycerols and cholesterol. The plasma markers for liver function, aminotransferases, alkaline phosphatases and bilirubin concentrations were all significantly suppressed by rooibos tea supplementation. The authors suggested that the natural antioxidants and scavenging agents in rooibos tea might be the hepato-protectors and could be included in the diet of patients with hepatopathies (375).

4.3 *Cyclopia intermedia*

4.3.1 History and processing

Honeybush tea (*Cyclopia intermedia*) is a traditional indigenous herbal beverage from South Africa. The plant belongs to the Cape fynbos biome grows only in the coastal districts of the Western and Eastern Cape Provinces from Darling to Port Elizabeth surrounded in the north by various mountain ranges. The earliest mention of the honeybush plant in botanical literature was in 1705 (376). It was also recognised by the Dutch and British colonists in the Cape as a suitable substitute for black tea, probably based on native practises for the treatment of coughs and other upper respiratory symptoms. The plant is a shrub of the *Fabaceae* family (*Leguminosae*) and is easily

recognised by its trifoliate leaves, single-flowered inflorescences and sweetly scented bright yellow flowers with prominent grooves on the standard petal. The honeybush plant has woody stems and a relative low ratio of leaves to stems with the leaf shape and size differing among the species. The name *Cyclopia* is derived from the Greek words *cyclos*, a circle and *pous*, a food, which allude to the intrusive base of the calyx (http://www.rooibos.ch/honeybush.html). Honeybush tea is prepared from the leaves, stems and flowers (Fig. 22) of several species of *Cyclopia*, including *C. sessiliflora*, *C. genistoides*, *C. subternata* and *C. intermedia*.

Commercial supplies of honeybush are mainly from *C. intermedia,* also referred to as "bergtee", meaning mountain tea, and to a lesser extent from *C. subternata* (377). The production of honeybush has grown in the past few years, from approximately 30 tons harvested in 1997 to 160 tons in 2000, with an estimate of 300 tons for 2004 (http://www.itmonline.org/arts/honeybush.htm). Although there has been a substantial growth in local consumption of honeybush tea, this increase mainly reflects the development of the international market as 90% of the annual honeybush production is exported (http://www.wesgro.org.za/-uploads/ssnaturalproducts_0800.pdf).

C. genistoides

C. intermedia

C. subternata

Fig. 22. Species of *Cyclopia* (Courtesy of Cape Natural Tea Products, Brackenfell, Cape Town, South Africa).

The honeybush industry is only in a developmental stage and consists mainly of processed *Cyclopia intermedia* harvested from natural plant populations. Cultivation has, however, become necessary. In 2000 the first large scale South African plantation dedicated to honeybush, began operation in the town of Haarlem, a subsistence farming community in the Langkloof, Eastern Cape Province. Due to its success, a second community at Ericaville, established a plantation in 2001 (Personal communication, Dawie de Villiers, Cape Natural Tea Products, South Africa). In response to the growing demand of honeybush tea, role players in the Honeybush Industry formed a representative body, SAHTA (South African Honeybush Tea Association) in 1999. Members include both growers as well as marketers.

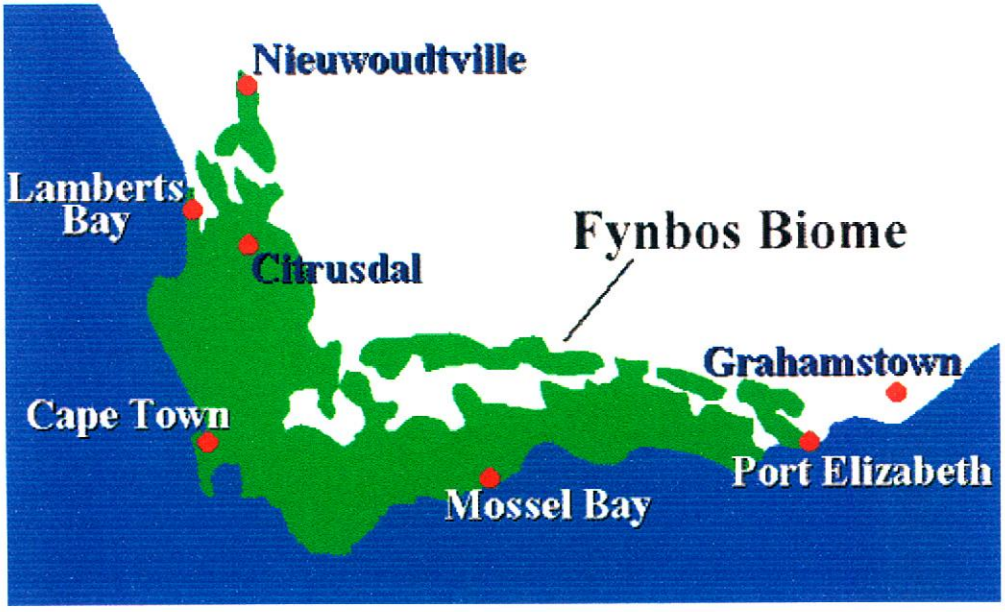


Fig.23. Map of Southern and Eastern Cape Provinces in South Africa illustrating the regions were honeybush is grown (http://www.itmonline.org/arts/honeybush.htm).

Traditionally, the bushes are harvested every two to three years during the flowering period which included September to October for *C. intermedia* and May to June for *C. sessiliflora,* with *C. subternata* being harvested annually. Due to the increasing demand, some of the producers are forced to extend their harvesting period. The bushes are often cut to the ground to reduce the occurrence of stems that die after harvesting (personal communication, Dawie De Villiers, Cape Natural Tea Products, South Africa). Two types of tea are manufactured, processed and unprocessed/"green" honeybush tea. The manufacture of the processed tea includes 4 steps, harvesting,

cutting, oxidation or "fermentation" and drying (Fig. 24). Mechanised fodder cutters are used to increase productivity and to deliver a more uniform product of plant material as it can vary between 6 mm to 3 cm. The cutting of the plant material is done to disrupt cellular integrity to facilitate the oxidation process.

Currently there are two methods for honeybush oxidation, e.g. "heap fermentation" and "oven fermentation". Heap fermentation is the traditional method used where cuttings of "green" honeybush material (1.5 to 2.5 tons) is firmly packed in a oval shaped heap (4 m by 1.5 m), covered with canvas bags and left for 3 days to allow spontaneous heat generation and oxidation. During this oxidation process the plant material changes from a green to dark brown colour and develops a sweet aroma. From the 3rd day onwards the heap is turned every 12 hrs and inspected after 3-5 days. After this oxidation process the heap is spread in a thin layer on canvas and allowed to dry in the sun. Oven oxidation is the preferred method as it yields a better and more consistent quality product. A preheated oven is used and a shorter oxidation period (24-36 hrs) is needed to obtain the processed form of the tea. The baking oven allows for reheating of about 385 kg of plant cuttings during the oxidation process without having to remove the plant material. Although sun drying is believed to enhance the final product's appearance, oven drying has been implemented by the bigger producers. The dried product is then sieved to remove all the undesirable bigger pieces, steam pasteurised and bulk packed in woven plastic bags for local and overseas markets.

A small portion is also packed in smaller sized bags for local sales. Honeybush tea is traditionally a very coarse product with the finer tea material used for teabags and the coarser material for brewing loose tea.

The unprocessed form is prepared by immediate drying of the cut plant material in a drying tunnel (40^{0}C for 12 hr), sieving and pulverization (378). The unprocessed honeybush tea only recently became part of the herbal tea market in South Africa (personal communication Elizabeth Joubert, Agricultural Research Council, Stellenbosch, South Africa) and its use for the preparation of an extract for the cosmeceutical market based on its antioxidant properties is under investigation.

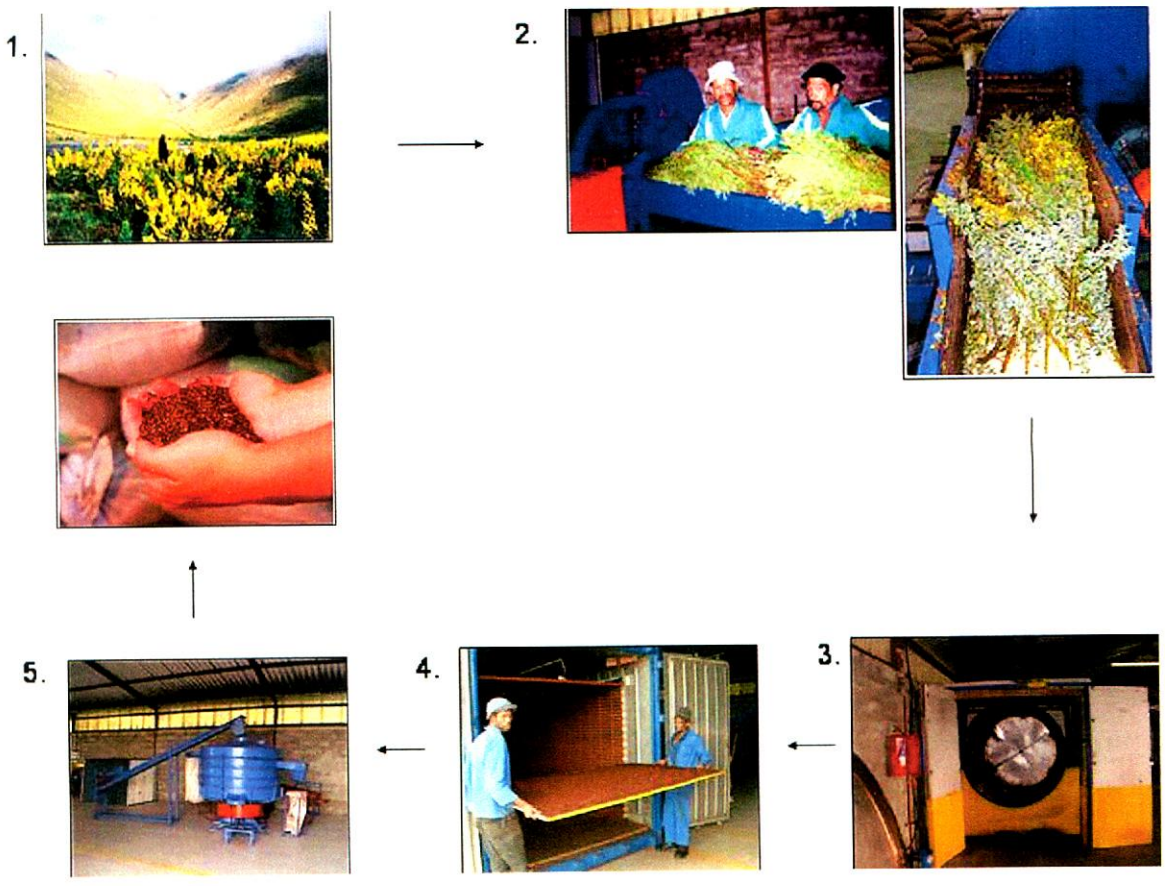


Fig. 24. Processing of honeybush plant material (Courtesy of Cape Natural Tea Products, Cape Town, South Africa).

4.3.2 Phenolic constituents

The major constituents in the leaves of various *Cyclopia* species were investigated by De Nysschen *et al.* (379). Honeybush tea differs from rooibos in the major classes of phenolic compounds, and three major constituents were identified in methanol extracts from the leaves of 22 *Cyclopia* species which include the xanthone, mangiferin and glycosides of the flavanones, hesperitin and isosakuranetin. A combination of these three compounds is a unique character for *Cyclopia* species and is present in varying quantities in the 22 species that were screened but no precise quantitative data could be obtained from the study (379).

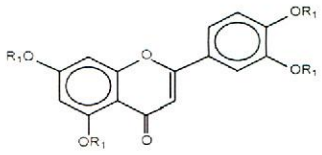
As *Cyclopia intermedia* is the main resource for local as well as export use, studies were conducted to identify phenolic compounds from this species (380,381). A methanol extract from the leaves and stems contained a phenolic acid, isoflavones, flavanones, coumestans and xanthones (Fig. 25). The phenolic acid was identified as 4-hydroxycinnamic acid, the isoflavones comprised of formononetin, afrormosin,

calycosin, pseudobaptigen and fujikinetin, the flavanones of hesperetin, hesperidin, eriodictyol, and naringenin, the coumestans of medicagol, flemichapparin and sophoracoumestan B and the xanthones of mangiferin and isomangiferin. The flavone luteolin and the inositol (+)-pinitol was also identified to be present in *C. intermedia* (380). Recently, a reversed-phase HPLC method was devised for the separation of the flavonoid fractions of four species of unprocessed *Cyclopia* in order to quantify the mangiferin, isomangiferin and hesperidin content in these plants (382). *C. intermedia* and *C. genistoides* contained the highest levels of mangiferin (3.6 % w/w), isomangiferin (0.54% w/w) and hesperidin (1.74% w/w). Harvesting dates, geographical location and processing, influences the phenolic composition of these plant extracts, e.g. chemical oxidation in the manufacturing of processed honeybush tea resulted in a significant decrease in mangiferin, isomangiferin, hesperidin (378,382).

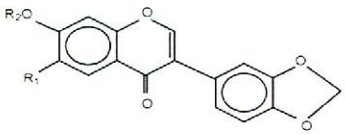
4.3.3 Biological activities

4.3.3.1 Anecdotal health claims

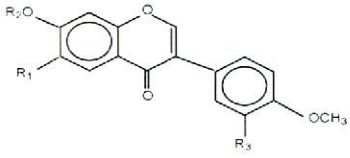
As for rooibos tea, honeybush tea also has very low tannin content and contains no caffeine (383,384) rendering it well suited for a night time beverage and for those experiencing nervousness. Several health benefits have been associated with regular consumption of this herbal tea, but these are mostly anecdotal. Traditionally honeybush tea is used as a diuretic, appetite stimulant, preventer of stomach ulcers, stimulator of milk production in lactating woman, treatment for colic babies and as a cough syrup (385-388). Researchers have shown high levels of (+)-pinitol to be present in honeybush (380,381). Pinitol has been used as an expectorant (389) and is known to possess anti-diabetic activity (390). Phytoestrogenic activity is also associated with isoflavonoids, coumestans, and the flavonoid groups, flavones and flavanones (391,392). These compounds have been shown to be present in *Cyclopia intermedia*. Phytoestrogens are used in the treatment of menopausal symptoms in women (393).



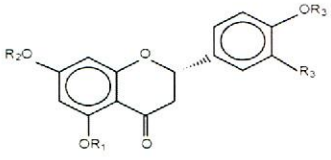
Flavones
Luteolin
R^1=H



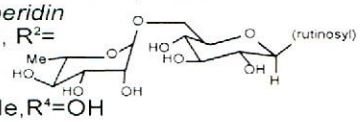
Isoflavones
Pseudobaptigen
R^1=R^2=H
Fujikinetin
R^1=OMe,R^2=H



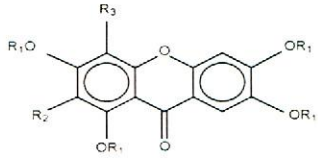
Isoflavones
Formononetin
R^1=R^2=R^3=H
Afromosin
R^1=OMe,R^2=R^3=H
Calycosin
R^1=R^2=H, R^3=OH



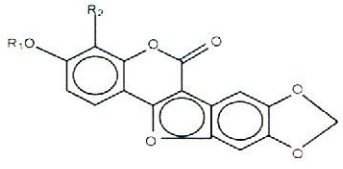
Flavanones
Naringenin
R^1=R^2=R^3=R^4=H
Eriodictyol
R^1=R^2=R^3=H, R^4=H
Hesperetin
R^1=R^2=H, R^3=Me, R^4=OH
Hesperidin
R^1=H, R^2=



R^3=Me,R^4=OH



Xanthones
Mangiferin
R^1=R^3=H, R^2=2-ß-D-glucopyranosyl
Isomangiferin
R^1=R^2=H, R^3=2-ß-D-glucopyranosyl



Coumestans
Medicagol
R^1=R^2=H
Flemichapparin
R^1=Me, R^2=H
Sophoracoumestan
R^1=H, R^2=OMe

Fig. 25. Major classes of phenolic constituents in *Cyclopia intermedia* (380).

4.3.3.2 Antioxidant activity

Thus far, very limited scientific data exist on the biological activities of this herbal tea. One such activity that has been established though is the *in vitro* antioxidant activity of various extracts as well as purified compounds (378,394). The oxidation process, during the manufacturing of processed tea, decreases the antioxidant activity of honeybush tea (378,394,395).

Antioxidant activity of honeybush tea has been demonstrated for aqueous extracts for several of the *Cyclopia* species *in vitro*. Hubbe (395) reported on the superoxide anion radical ($O_2^{\cdot -}$) scavenging activity as well as the hydrogen donating ability of four *Cyclopia* species, *C. intermedia*, *C. sessiliflora*, *C. genistoides* and *C. subternata*. Unprocessed *C. sessiliflora* was the most effective scavenger of both the $DPPH^{\cdot}$ radical as well as the superoxide anion radicals. This species was also shown to have the highest polyphenol content. Using a linoleic acid buffer system, the inhibitory effect of the various honeybush species on lipid peroxidation was also determined. The processed and unprocessed counterpart of *C. genistoides* exhibited the highest protection in this assay (395). Subsequent to this study, Richards (378) further investigated the antioxidant activity of the various honeybush species using ABTS and FRAP assays for radical scavenging ability and a microsomal lipid peroxidation model, measuring the inhibition of MDA formation *in vitro*. Overall, unprocessed *C. intermedia* and *C. sessiliflora* showed the highest antioxidant activities, with *C. genistoides* being the most potent for the processed honeybush plant material (378).

Further *in vitro* investigations into the antioxidant activity of various honeybush tea extracts and purified compounds are currently under investigation at ARC Infruitec-Nietvoorbij, Stellenbosch under leadership of Dr E Joubert.

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CHAPTER 3

An Investigation on the Antimutagenic Properties of South African Herbal Teas

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An Investigation on the Antimutagenic Properties of South African Herbal Teas

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ABSTRACT

The antimutagenic properties of South African herbal teas were investigated using the *Salmonella typhimurium* mutagenicity assay. Aqueous extracts of fermented and unfermented rooibos tea (*Aspalathus linearis*) and honeybush tea (*Cyclopia intermedia*) both possess antimutagenic activity against 2-acetylaminofluorene (2-AAF) and aflatoxin B_1 (AFB_1)-induced mutagenesis using tester strains TA 98 and TA 100 in the presence of metabolic activation. A far less inhibitory effect was noticed against the direct acting mutagens, methyl methanesulfonate (MMS), cumolhydroperoxide (CHP), and hydrogen peroxide (H_2O_2) using TA 102, a strain designed to detect oxidative mutagens and carcinogens. Depending on the mutagen used, the unfermented tea exhibited the highest protective effect. A similar response regarding the protection against mutagenesis was obtained when utilizing different variations of the double layer *Salmonella* assay. The double layer technique proved to be more effective to detect the protective effect of the different tea preparations against the direct acting mutagens. With respect to indirect mutagens, the highest protection was noticed when the carcinogen was metabolically activated in the presence of the tea extract as compared with when the tea extract was incubated in a separate layer with the bacteria. The current data suggest that two mechanisms seem to be involved in the antimutagenicity of the tea extracts towards carcinogens that require metabolic activation: (i) the tea components may interfere with cytochrome P450-mediated metabolism of these mutagens and (ii) the direct interaction between the tea constituents, presumably the polyphenolic compounds, with the promutagens and/or the active mutagenic metabolites. However, the mild and/or lack of protection and in some cases even enhancement of mutagenesis induced by direct acting or oxidative mutagens, provide new perspectives regarding the role of the polyphenolic compounds known to exhibit

antioxidant properties, in the protection against mutagenesis in the *Salmonella* assay. The present study provides the first evidence on the antimutagenic activity of honeybush tea and further evidence on the antimutagenicity of rooibos tea.

KEYWORDS: Rooibos tea; Honeybush tea; Antimutagenicity; *Salmonella* assay.

INTRODUCTION

Epidemiological data suggest that more than 80% of cancers are attributable to lifestyle, of which at least one third is diet-related [1]. Diets containing substances that can modulate or prevent cancer may play a role in the general health of humans, especially if they are inexpensive and easily available. Tea is a widely consumed beverage throughout the world while the popularity of herbal health teas increased significantly during the past twenty years [2]. The anticancer properties of tea are well known, and the tumour inhibition potential of certain polyphenolic compounds from green and black tea has been well documented [3-7].

Both rooibos tea (*Aspalathus linearis*) and honeybush tea (*Cyclopia intermedia*), two South African herbal teas, have been shown to contain a complex mixture of polyphenolic compounds, of which aspalathin, a dihydrochalcone, is unique to rooibos tea while luteolin is one of the most important flavones in honeybush tea [8–10]. Processing of these teas include the following steps, fermentation of leaves and stems, followed by sun-drying and sieving and in the case of rooibos tea, steam pasteurization of the dried product before packaging [11,12]. Rooibos tea is popular as a health beverage, as it contains no harmful stimulants and no caffeine with only trace amounts of tannins [13,14]. Extracts of rooibos tea have been found to decrease the number of chromosome aberrations when treating Chinese hamster ovary (CHO) cells with benzo[*a*]pyrene (B[*a*]P) in the presence of metabolic activation [15]. An *in vivo* study in male ICR mice indicated that the number of micronucleated reticulocytes (MNRET) induced by single dosage of mitomycin C (MMC) was significantly reduced by daily ingestion of rooibos tea. However, a single gavage treatment of rooibos tea 24 h before MMC injection did not reduce MNRET frequency [15]. Suppression of oncogenic transformation of mouse CH310T1/2 cells induced by X-rays in the presence of rooibos tea was also shown in a study by Komatshu *et al.* [16]. However, green tea extracts at an equitoxic concentration did not show any detectable suppression [16]. In a study done by Standley [17], it was reported that water soluble fractions of both fermented and unfermented rooibos tea possess antimutagenic activity against 2-

acetylaminofluorene (2-AAF)-induced mutagenesis using strain TA 98 in the *Salmonella* mutagenicity assay. Apart from these studies on rooibos tea very little is known about the antimutagenic and anticarcinogenic properties of the two South African herbal teas. Most of the research regarding the antimutagenicity and anticancer properties of tea, as well as chemoprevention and epidemiological studies in humans to date has focused on green and black teas [18–24]. Most of these studies indicated that polyphenol preparations and/or water extracts from green and black tea dramatically decreased the mutagenicity of a variety of genotoxic carcinogens. As damage to DNA is likely to be a major cause of cancer and other chronic diseases [25,26], the protective effect of naturally occurring dietary constituents could effectively reduce the onset and/or progression of cancer.

The *Salmonella* mutagenicity assay has been extensively used to monitor the mutagenic potential of a variety of compounds that either occur naturally or are introduced artificially into the environment by various means [19,22,27,28]. In the present study, this test system was utilized to monitor the antimutagenic properties of aqueous extracts of fermented and unfermented rooibos and honeybush tea against a variety of direct-acting mutagens as well as two indirect mutagens that required metabolic activation.

MATERIALS AND METHODS

Chemicals and media. The mutagens, 2-AAF and aflatoxin B_1 (AFB_1) were purchased from Sigma Chemical Co. (SA) at the highest available purity. Cumolhydroperoxide (CHP) was purchased from Merck (Schuchardt, Germany), hydrogen peroxide (H_2O_2) from Saarchem (SA) and methyl methanesulfonate (MMS) from Aldrich Chemical Co. (SA). Stock solutions of the different carcinogens were freshly prepared on the day of the experiment in dimethyl sulfoxide (DMSO) purchased from BDH Laboratory Supplies (Poole, UK). All other solvents used were of analytical grade. Agar and Nutrient Broth No. 2 were purchased from the Difco Laboratories (Detroit, USA) and Oxoid (Hampshire, UK), respectively.

Antimutagenicity assay

Standard plate incorporation assay. Antimutagenicity of the tea extracts against different mutagens was assessed using the standard plate incorporation assay as described by Maron and Ames [29]. *Salmonella typhimurium* strains TA 98, TA 100 and

TA 102 were kindly provided by Dr. B.N. Ames (Berkely, CA). For the plate incorporation inhibition assay, 0.1 ml of each mutagen, 0.1 ml of the various tea extracts, 0.5 ml S9-mix and 0.1 ml of an overnight bacterial culture were carefully mixed with 2 ml of molten top agar, containing 0.05 mM biotin–histidine and dispersed onto minimal glucose agar plates. A liver S9 homogenate (0.72 nmol cytochrome P450/mg protein) was prepared by inducing male Fischer rats (200 g) with aroclor-1245 as described by Maron and Ames [29]. The S9 homogenate was incorporated into the S9-mixture at a level of 2 mg protein per ml. The mutagenicity of 2-AAF (5 μg per plate) and AFB_1 (10 ng per plate) were monitored against TA 98 and TA 100 in the presence of the S9 mixture, respectively. The direct acting mutagens were tested against strain TA 102: MMS (10 μM per plate), CHP (400 μg per plate) and H_2O_2 (1.81 mg per plate) in the absence of the S9 mixture. A series of control plates containing only the tea extracts and the bacteria in the absence and presence of the S9 mixture were also included to screen the different tea preparations for mutagenic effects. Control plates containing only DMSO, which was used as the solvent vehicle, were also included to obtain the background or spontaneous revertant counts.

Double layer assay. A modification of the double layer technique described by Glatt and Oesch [30] was also performed using two direct acting mutagens, MMS and CHP and one requiring metabolic activation (2-AAF). Two variations of the technique were used, one where the carcinogen and the tea preparation were included in the lower top agar layer, with the bacterial suspension in the upper top agar layer (referred to as α) and another where the carcinogen was included in the lower top agar layer with the tea preparation and bacterial suspension in the upper top agar layer (referred to as β). Two concentrations of the tea extracts (5 and 10% (w/v)) were used with MMS (β technique) while only a 10% (w/v) concentration was used with CHP and 2-AAF (α technique).

All plates were incubated at 37 ^{0}C for 48 h. Thereafter, the histidine revertants were counted using a Quebec Colony Counter (American Optical Corp., Buffalo, New York). All the experiments were repeated once, and five replicates were included for each sample.

Preparation of tea extracts. Fermented and unfermented rooibos and honeybush tea were used. Aqueous extracts of the teas were prepared by the addition of freshly boiled

water to the tea leaves and stems to a concentration of 2 g/100 ml for rooibos tea and 4 g/100 ml for honeybush tea. These concentrations are customarily used for tea making purposes [11,12]. The mixture was allowed to stand for 30 min at room temperature, filtered (Whatman No. 4), freeze-dried and stored in airtight containers at −20 ^{0}C. Two different tea solutions (5 and 10% (w/v)) were prepared from the freeze-dried extracts in distilled water, centrifuged at 300 rpm and the supernatant sterilized through 0.45 and 0.22 μm filters. A methanol extract of unfermented rooibos tea was also prepared following excessive chloroform extraction. Thereafter, the solvent was evaporated at 48 ^{0}C *in vacuo*. The protective effect of the methanol extract (5 and 10% (w/v) in DMSO) was also monitored against the different mutagens.

Soluble solid and polyphenol determination. The soluble solid content of each tea preparation (2 and 4% for rooibos and honeybush tea, respectively) was determined gravimetrically after drying 1 ml aliquots at 110 ^{0}C for 12 h. Five determinations for each tea preparation were done. The scaled-down Folin–Ciocalteau assay was used to determine the total phenolic content [31] of the tea extracts (performed in triplicate), while non-flavonoids were determined after precipitation of flavonoids [32].

Statistical analysis. Analysis of variance (ANOVA) was performed using the statistical analysis system (SAS) programme. The Tukey *T*-test was used to determine whether the means of the control and positive control groups differed significantly. The Dunnett test was used to determine whether the means of the tea treatments differed significantly from the positive mutagenic control treatments. Bonferroni pairwise adjustment was used to compare the soluble solids, total polyphenols, flavonoids and non-flavonoids of the fermented and the unfermented tea preparations.

RESULTS

Analytical data. The soluble solids, total polyphenol, flavonoid and non-flavonoid content of the different tea extracts used in this study are summarized in Table 1. Changes in the total polyphenol and flavanoid content of the two teas during processing are clearly noted, as they were significantly ($P < 0.001$) reduced during the fermentation process. The soluble solids were significantly higher in the honeybush tea extract, which is in agreement with the larger amount of the tea used in the preparation of the extract.

Table 1 Soluble solid matter and total polyphenols in the different tea preparations[a]

Tea extracts	Total polyphenols (%)[b]	Flavonoids (%)[b]	Non-flavonoids (%)[b]	Solid matter (mg/ml)[c]
Unfermented rooibos tea (2% w/v)	41.15 ± 0.25ad	28.06 ± 0.25ad	13.10 ± 0.04ad	4.21 ± 0.44ad
Fermented rooibos tea (2% w/v)	29.74 ± 0.36Ad	18.8 ± 0.35Ad	10.94 ± 0.05Ad	3.42 ± 0.22ad
Unfermented honeybush tea (4% w/v)	35.52 ± 0.03b	27.10 ± 0.02b	8.42 ± 0.05b	11.99 ± 1.09b
Fermented honeybush tea (4% w/v)	19.80 ± 0.26B	9.86 ± 0.18B	9.94 ± 0.08B	5.26 ± 0.37c

[a] Values ± SD are the mean of triplicate determinations. Statistical comparisons were made within a group. Means in columns followed by the same letter do not differ significantly. If the letters differ then $P < 0.01$. When the cases differ then $P < 0.001$. [b]Soluble solid matter (g per 100 g). [c]Aqueous tea extracts. [d]Indicates significant differences between the two tea groups (i.e. unfermented rooibos vs. unfermented honeybush and fermented rooibos vs. fermented honeybush).

Antimutagenicity of tea extracts. The protective effect of the different tea extracts on the mutagenicity of the various chemical mutagens and carcinogens using the plate incorporation assay are summarized in Table 2. Data on the protective effect of the different tea extracts against the mutagenicity of the various compounds utilizing the double layer technique are presented in Table 3. For data analyses the values of the two separate experiments were combined, as it did not differ statistically.

Mutagens requiring metabolic activation

2-Acetylaminofluorene. Addition of fermented and unfermented rooibos and honeybush tea significantly ($P < 0.001$) reduced 2-AAF-induced mutagenesis. A clear dose response effect was noticed with the 10% (w/v) extract exhibiting the highest protective effect with both teas. A similar protective effect was obtained with the methanol extract of the unfermented rooibos tea at both concentrations, almost completely inhibiting 2-AAF-induced mutagenesis. At the 5% concentration level the fermented rooibos tea exhibited a higher protective effect than the unfermented counterpart. However, at the 10% concentration level there was no significant difference between the unfermented and fermented rooibos preparations. The unfermented honeybush tea preparation exhibited a significantly higher protective effect than the fermented tea at both concentration levels. When compared with fermented honeybush,

the fermented rooibos tea exhibited a higher protective effect, whereas unfermented honeybush tea (5%) exhibited a higher protective activity than its rooibos counterpart. The total polyphenolic content related well to the protective effect exhibited by the different tea preparations as a higher total polyphenolic concentration is associated with a higher protective effect (Table 2).

Aflatoxin B_1. As described for 2-AAF the addition of both the 5 and 10% fermented and unfermented rooibos tea, significantly ($P < 0.001$) reduced the number of revertants induced by AFB_1 with the unfermented tea exhibiting a higher protective activity (Table 2). A similar protective effect (with no significant difference between fermented and unfermented) was obtained with honeybush tea at both concentration levels. No dose response effects were noticed with either rooibos or honeybush tea. In contrast to the effect on 2-AAF-induced mutagenesis, no difference could be detected between the protective effect of fermented and unfermented rooibos and honeybush tea. The methanol extract again showed a similar protective effect when compared with the unfermented rooibos tea preparations lacking any dose response effects. In the case of AFB_1-induced mutagenicity the higher total polyphenolic content did not relate with the higher protective effect.

Direct acting mutagens

Cumolhydroperoxide. Only fermented rooibos tea (10%) and unfermented honeybush tea (10%) preparations showed a significant ($P < 0.05$) decrease in the CHP-induced mutagenesis (Table 2). The methanol extract and the unfermented rooibos (10%) also slightly reduced the mutagenic response, but the reduction was not significant. Fermented honeybush tea (5%) tended to increase the number of revertant colonies in the presence of CHP, although not significantly. Only the unfermented honeybush tea preparation showed a higher protective effect associated with a dosage increase from 5 to 10%, which related well to the higher total polyphenolic content.

Hydrogen peroxide. Preparations of fermented (10%) and unfermented (10%) rooibos tea and the methanol extract (10%) showed significant decreases ($P < 0.05$ and $P < 0.001$) in the number of histidine revertants (Table 2). The total polyphenolic content of the rooibos tea preparations were in relation with the protective effect as the 5% rooibos tea preparation showed a far less inhibitory effect. No protective effect was noticed with fermented and unfermented honeybush tea although the 10% (w/v)

unfermented honeybush tea also showed a slight (non-significant) protective effect.

Methyl methanesulfonate. No protective effect was noticed with the different tea extracts, while the methanol extract of unfermented rooibos tea (5%) significantly ($P < 0.05$) reduced MMS-induced mutagenesis. On the other hand, unfermented rooibos tea (10%) significantly ($P < 0.001$) enhanced the revertant counts.

The double layer technique

When utilizing the double layer technique (β = MMS in lower layer of top agar, with the tea preparation and TA 102 in the top layer) the methanol extract markedly and significantly reduced the number of MMS-induced revertants when incorporated at 5 ($P < 0.05$) and 10% ($P < 0.001$), respectively. In contrast to the plate incorporation assay, both the unfermented rooibos ($P < 0.001$) and honeybush ($P < 0.05$) tea (10%) significantly reduced the revertant counts (Table 3), respectively. No significant protective effect was noticed with the fermented counterparts. With respect to CHP no significant differences between the two variations of the double layer technique (α = CHP and tea in bottom layer and TA 102 in top layer and β = CHP in bottom layer and tea and TA 102 in top layer) were noticed. When compared with the standard plate incorporation assay the methanol extract ($P < 0.05$) and both the fermented rooibos preparations (5 and 10%) ($P < 0.001$) and the unfermented honeybush (10%) ($P < 0.05$) showed significant reductions in the revertant counts induced by CHP (Table 3).

In the case of the metabolically activated carcinogen, 2-AAF, both tea preparations significantly ($P< 0.001$) reduced the revertant colonies in both variations of the test. However, the α-method resulted in a significantly ($P<0.001$) higher protection against mutagenesis than the β-method (Table 3).

Control tea treatment plates. No toxicity or mutagenic activity of the different tea extracts was detected in the presence of metabolic activation at the levels used in the *Salmonella* test (data not shown). The spontaneous revertant counts of the different strains in the presence and absence of DMSO were in the range of published values [29].

Table 2 Protective effects of aqueous rooibos and honeybush tea extracts on mutagenicity of a variety of carcinogens used in the *Salmonella* assay[a]

Treatments	Total polyphenols	Revertants per plate				
Carcinogen	(mg per plate)b	TA 98	TA 100	TA 102		
		2-AAF	AFB_1	CHP	H_2O_2	MMS
		5 μg per plate	10 ng per plate	400 μg per plate	1.81 mg per plate	(10 μM per plate
Control (−)		32 ± 5	120 ± 6	176 ± 18	317 ± 22	183 ± 16
Control (+)		513 ±14a	309 ± 22a	870 ± 144a	950 ± 122a	535 ± 34a
RBf (5%)	1.49	150 ± 29B	158 ± 21B	784 ± 66a	1028 ± 63a	592 ± 66a
RBf (10%)	2.97	88 ± 12C	145 ± 6B	614 ± 67b	712 ± 81b	577 ± 53a
RBu (5%)	2.06	272 ± 30D	128 ± 6CD	850 ± 100a	858 ± 146a	635 ± 62a
RBu (10%)	4.12	43 ± 6C	113 ± 11CD	702 ± 37a	697 ± 91B	676 ± 46B
Me (5%)	ND	40 ± 4C	115 ± 6C	702 ± 68a	805 ± 116a	442 ± 77b
Me (10%)	ND	30 ± 7C	109 ± 10C	706 ± 62a	650 ± 145b	522 ± 44a
HBf (5%)	0.99	318 ± 26D	157 ± 6B	926 ± 79a	968 ± 131a	602 ± 64a
HBf (10%)	1.98	182 ± 28E	131 ± 16BD	832 ± 68a	945 ± 59a	616± 43a
HBu (5%)	1.78	143 ± 16B	139 ± 13BD	854 ± 116a	1012 ± 33a	562 ± 34a
HBu (10%)	3.55	68 ± 12C	125 ± 14BD	674 ± 62b	805 ± 155a	606 ± 63a

[a] Values are mean ± S.D. of five replications. Means in columns followed by different letters then $P < 0.05$, when the cases differ then $P < 0.001$. RBf, rooibos fermented tea; RBu, rooibos unfermented tea; HBf, honeybush fermented tea; HBu, honeybush unfermented tea; Me, methanol extract of rooibos unfermented tea (DMSO as solvent); ND, not determined. [b] Calculated from the mean total polyphenol content of the respective tea preparations presented in Table 1.

Table 3 Effect of aqueous rooibos and honeybush tea extracts on mutagenicity of a variety of carcinogens in the double layer *Salmonella* assay[a]

MMS (10 μM per plate)		CHP (400 μg per plate)		2-AAF (5 μg per plate)	
Treatments	**TA 102 revertants/plate**	**Treatments (10%)**	**TA 102 revertants/plate**	**Treatments (10%)**	**TA 98 revertants/plate**
Control (−)	170 ± 16	Control (−)	214 ± 21	Control (−)	28 ± 3
Control (+)	522 ± 39a	Control (+)	1065 ± 134a	Control (+)	450 ± 29a
RBf (5% β)	507 ± 58a	RBf-α	702 ± 83A	RBf-α	119 ± 18Ab
RBf (10% β)	443 ± 30a	RBf-β	730 ± 174A	RBf-β	190 ± 32A
RBu (5% β)	453 ± 44a	RBu-α	960 ± 108a	RBu-α	40 ± 6Ab
RBu (10% β)	395 ± 60A	RBu-β	752 ± 167a	RBu-β	67 ± 8A
Me (5% β)	442 ± 77a	Me-α	787 ± 77b	Me-α	ND
Me (10% β)	406 ± 17A	Me-β	725 ± 73b	Me-β	ND
HBf (5% β)	498 ± 45a	HBf-α	1295 ± 48a	HBf-α	32 ± 2Ab
HBf (10% β)	489 ± 47a	HBf-β	1195 ± 149a	HBf-β	69 ± 12A
HBu (5% β)	502 ± 24a	HBu-α	940 ± 42a	HBu-α	32 ± 4Ab
HBu (10% β)	423 ± 38b	HBu-β	813 ± 32b	HBu-β	79 ± 6A

[a]Values are the means of five determinations ± S.D.: α, carcinogen and/or S9 and tea extract in bottom layer, bacterial strain in top layer; β, carcinogen and/or S9 in bottom layer, bacterial strain and tea extract in top layer. Means in columns followed by different letters then $P < 0.05$, when case differ then $P < 0.001$. RBf, rooibos fermented tea; RBu, rooibos unfermented tea; HBf, honeybush fermented tea; HBu, honeybush unfermented tea; Me, methanol extract of unfermented rooibos tea (DMSO as solvent); MMS, methyl methanesulfonate; CHP, cumolhydroperoxide; 2-AAF, 2-acetylaminofluorene; ND, not determined.
[b]$P < 0.001$ (differences between α and β variations).

DISCUSSION

Five different mutagens with diverse chemical structures and mode of action were used to determine the protective effect of two South African herbal teas against their mutagenicity. Aqueous extracts of unfermented rooibos and honeybush teas displayed a strong antimutagenic effect against both of the metabolically activated carcinogens, 2-AAF and AFB_1. In general the fermented tea preparations showed a high level of protection, but not as effective as their unfermented counterparts with the exception of 5% fermented rooibos tea, which exhibited a higher protective effect against 2-AAF-induced mutagenesis. In the case of 2-AAF, the fermented and unfermented rooibos (10%) tea preparations exhibited a higher inhibition potential than the corresponding honeybush tea preparations. When comparing the protection of the two herbal teas against AFB_1-induced mutagenicity, they showed a very similar protective effect despite the fact that no dose response effects were noticed, suggesting that a threshold was

reached with the 5% tea preparation. As discussed above, the unfermented rooibos tea extract exhibited a higher protective effect than the fermented counterpart, whereas no such effect was noticed with the different honeybush tea preparations.

When considering the protective potency of the teas against mutagens that require metabolic activation, cognizance has to be taken of the fact that the different tea preparations were tested on the same weight basis. The total polyphenol content of the extracts is clearly more relevant for comparative purposes. The unfermented teas contain higher concentrations of polyphenolic compounds, which are significantly reduced during the fermentation process, due to oxidation [33]. In the case of 2-AAF, these oxidized compounds could either have a lower antimutagenic potential or have lost their antimutagenic potential, as the total polyphenol content of the various tea preparations corresponded well with protection against mutagenesis. No relation with the polyphenol content was noticed when comparing the protective effect of unfermented and fermented rooibos at the 5% level. When AFB_1 was used as the mutagen, unfermented rooibos tea exhibited a higher protective effect than the fermented tea, which again related well with the higher polyphenol content. However, both the unfermented and fermented honeybush tea exhibited a similar effect indicating that the oxidation of compounds during fermentation does not reduce the protective effect. Furthermore, fermented rooibos and honeybush tea exhibited similar protective effects whilst the unfermented rooibos tea exhibited a higher level of protection than honeybush tea. These data suggest that, when metabolic activation is required, differences exist in the degree of protection that not only depend on the type of tea and/or polyphenol content but also on the specific mutagen used.

The role of antioxidants has attracted much interest with respect to their protective role against free radical damage that may be the cause of many diseases including cancer [34]. The antioxidative effect of green tea is mainly due to the phenolic components, such as the flavonoids [35]. Some flavonoid and non-flavonoid phenolic compounds have been reported to also show alkylperoxyl radical scavenging activity thus reducing radical-mediated pathogenesis, e.g. carcinogenesis [36]. In the present study only a weak relation exists between the polyphenolic content of the herbal teas and the antimutagenic effect when using the oxidative mutagens, H_2O_2, and CHP and the direct acting mutagen MMS. Other investigators [18,37,38] have also noted this failure and/or weak protective effect against direct acting mutagens. Constable *et al.* [37] reported that the major polyphenols are not the only compounds responsible for the protective effects

of the green and black teas seen in the bacterial mutagenicity assay. As a reduction of the catechin content in fermented and instant teas did not correlate with the antimutagenic effects observed in the assay, other compounds formed during the processing conditions may be involved. This may well be the case for the SA herbal teas where the high oxidative properties of the fermented and unfermented teas did not correspond well with the low protective effect against the various oxidative mutagens used in the present study.

As discussed for the metabolically activated mutagens, differences exist with respect to the protective effect of the tea preparations and specific mutagen used. No protective effect was noticed against MMS, whereas with H_2O_2 fermented and unfermented rooibos tea (10%), and with CHP, fermented rooibos and unfermented honeybush (10%) tea, showed a protective effect. Only a weak relationship exists with respect to the polyphenolic content of the teas and the protective effect against the direct acting mutagens. Both the fermented and unfermented rooibos tea preparations (10% w/v) showed a similar inhibition when using H_2O_2 as direct-acting mutagen despite the fact that the total polyphenols was significantly lower in the fermented tea preparation. With CHP only the highest concentrations of the fermented rooibos and unfermented honeybush tea exhibited a protective effect. In some cases (CHP and H2O2), the addition of the tea preparations (except the methanol extract) resulted in a marked (non-significant) increase in the mutagen-induced response. One possible explanation for this weak/lack of protection against the direct acting mutagens could be that the *in vivo* induction of antioxidative enzymes is required to protect against oxidative-like mutations. Another aspect that needs to be considered is the polarity of the mutagen used compared with that of the protective principles in the aqueous tea preparations. The relatively polar constituents (including some polyphenols) of the aqueous extracts could be responsible for the low protective properties against direct acting mutagens. Due to the differences in polarity, molecules can be restricted to either hydrophilic or hydrophobic environments that will minimize their possible interaction. For example, the polar constituents of the aqueous extract will be associated with the polar constituents in the medium whilst the non-polar mutagens, MMS and CHP, are likely to be associated with the bacterial cellular matrixes. This became evident as a higher protective effect of the tea was obtained when incubating the fermented rooibos tea and the methanol extract with TA102 (top layer) and the mutagen (CHP) in the bottom layer, which increased the possibility of the tea constituents to interact with the direct acting mutagen.

With respect to the mutagens that required metabolic activation, the results obtained from the double layer assay provided insight into the possible mechanisms involved in the antimutagenic effects of the tea extracts. A higher degree of protection is obtained when 2-AAF, the S9 mixture and the different tea preparations are incubated in the bottom soft agar layer and TA98 in the top agar layer (α-method) than when the tea is incubated with the bacterium (β-method). In both cases, the activated metabolite has to diffuse from the bottom to the top agar layer to interact with the bacterium. To be mutagenic, 2-AAF, has to be metabolically activated by the cytochrome P450 enzyme system located in a hydrophobic pocket in the microsomes [39]. When the tea extracts are incubated with the bacterium the protective effect could be ascribed to a direct interaction of the activated mutagenic metabolite that diffuses from the bottom layer. However, when the tea is incubated with the enzyme preparation in the bottom layer, the inhibitory effect on 2-AAF mutagenesis can result from an interaction between the different components of the tea extracts and the enzyme system catalyzing the metabolic activation of the various promutagens (inhibiting cytochrome P450-mediated activation of the carcinogen) and thus impeding the production of genotoxic intermediates. Tea polyphenols can serve as such electron acceptors directing the flow of electrons from NADPH away from cytochrome P450, the terminal component in the electron-transport chain [38]. The structure of flavanols (also referred to as catechins), the major polyphenol in green and black tea, provide strong nucleophilic centres which enables them to react with electrophilic carcinogens to form flavanol-carcinogen adducts that may result in the prevention of tumorigenesis [40,41]. This ability of aqueous green tea extracts to function as nucleophiles is supported by Bu-Abbas *et al.* [38]. Rooibos tea contains 4.4% tannins [13] consisting of flavanol chain extending units [42]. Unlike green and black tea, rooibos tea also contains other polyphenols like flavonols (*iso*-quercitrin), flavones (orientin, *iso*-orientin, luteolin) and dihydrochalcones (aspalathin, nothofagin) [33]. Honeybush tea has been shown to contain flavones (luteolin) and flavanones (hesperidin, hesperetin, naringenin, eriodictyol) [8]. The electron-rich aromatic B-ring system of the flavones can supply electrons that are required for the reduction of the active oxygen species [33]. These phenolic compounds presumably contribute towards the scavenging ability of the herbal teas. Presently, it is unknown whether all these different polyphenols may play a role in the antimutagenic activity of rooibos and honeybush tea. Apart from the interference with the metabolic activating system, the tea components could also directly interact with the genotoxic reactive intermediates that may result in the prevention of

mutagenesis as discussed for the difference in protection obtained with b-method.

The antimutagenic effects of the different rooibos and honeybush tea preparations reported in this study could, in the case of 2-AAF that required metabolic activation, be related to the action of the different polyphenols. Studies are in progress to elucidate the mechanisms of antimutagenicity and to characterize the protective principles produced by these indigenous teas. It is known that different causative factors (e.g. carcinogens) are involved in the development of different cancers suggesting different mechanisms, which is organ specific. The present study indicated that the mechanism of protection obtained with the herbal teas differs not only when using direct-acting mutagens but also differs between mutagens that require metabolic activation. It can be argued that the herbal teas could protect against the activity of diverse mutagenic and possible carcinogenic compounds *in vivo* and their effectiveness, as potential chemopreventive drugs will depend on the mechanism of cancer development in a specific organ. In conclusion, it would appear that the herbal teas may not only be a good dietary source of natural antioxidants to counteract the damaging effects of free hydroxyl, superoxide and peroxyl radicals *in vitro* [31,43], but may also protect against mutagenesis.

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CHAPTER 4

Modulation of Hepatic Drug Metabolizing Enzymes and Oxidative Status by Rooibos (*Aspalathus linearis*) and Honeybush (*Cyclopia intermedia*), Green and Black (*Camellia sinensis*) Teas in Rats

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Modulation of Hepatic Drug Metabolizing Enzymes and Oxidative Status by Rooibos (*Aspalathus linearis*) and Honeybush (*Cyclopia intermedia*), Green and Black (*Camellia sinensis*) Teas in Rats

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ABSTRACT

Rooibos and honeybush teas significantly ($P < 0.05$) enhanced the activity of cytosolic glutathione S-transferase alpha. A significant ($P < 0.05$) to marginal ($P < 0.1$) increase in the activity of the microsomal UDP-glucuronosyl transferase was obtained with unprocessed rooibos and honeybush teas, respectively. Oxidized glutathione (GSSG) levels were significantly ($P < 0.05$) reduced in the liver of all tea treated rats while reduced glutathione (GSH) was markedly increased in the liver of the herbal tea treated rats. These changes resulted in a significant ($P < 0.05$) increase in the GSH/GSSG ratio by the unprocessed, processed rooibos and unprocessed honeybush teas. Green and black teas markedly to significantly decreased the oxygen radical absorbance capacity in liver homogenates, respectively. Modulation of phase II drug metabolizing enzymes and oxidative status in the liver may be important events in the protection against adverse effects related to mutagenesis and oxidative damage.

KEYWORDS: Rooibos (*Aspalathus linearis*) tea; honeybush (*Cyclopia intermedia*) tea; glutathione S-transferases; UPD-glucuronosyl transferases; glutathione; ORAC; rat liver

ABBREVIATIONS USED

CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 3,4-dichloro-nitrobenzene; GSH, reduced glutathione; GSSG, oxidized glutathione; UDP-GT, uridine 5′-diphospho-glucuronosyl-transferase; GST, glutathione S-transferase; M2VP, 1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; β-PE, β-phycoerythrin; ORAC, oxygen radical absorbance capacity; DPPH, 1,1-diphenyl-2-picrylhydrazyl.

INTRODUCTION

The protective role of tea (*Camellia sinensis*) against mutagenesis and carcinogenesis has been well established in the literature (*1-5*). Similar information regarding the protective role of the South African herbal teas, *Aspalathus linearis* (rooibos tea) and *Cyclopia* species (honeybush tea), is being established (*6*). These herbal teas, prepared from the indigenous Cape "fynbos" plants are consumed both locally and abroad with global consumption of rooibos composing more than 50% of its annual production of 7500 tons (http://www.emg.org.za/ documents/rooibos.pdf), while 90% of honeybush annual production of 150 tons is exported (http://www.wesgro.org.za/ uploads/ssnatural•products•0800.pdf). The honeybush industry is only in a developmental stage and consists mainly of processed *Cyclopia intermedia* harvested from natural plant populations. Apart from these traditional processed herbal teas where production includes an oxidation step/"fermentation" (*7, 8*), the manufacture of unprocessed ("green") rooibos and honeybush teas has recently commenced. During this process, oxidation of the polyphenols is minimized to obtain a product with enhanced antioxidant and antimutagenic activity (*9, 10*).

Several health-promoting properties have been associated with the consumption of these herbal teas, but evidence is mostly anecdotal (*11*). Limited research has been conducted regarding the biological effects of these teas. Extracts of rooibos tea exhibited antimutagenic activity in Chinese hamster ovary (CHO) cells and in male ICR mice (*12*). Recent studies in the *Salmonella* mutagenicity assay showed that aqueous extracts of unprocessed ("unfermented") and processed ("fermented") rooibos and honeybush teas prevent mutagenesis induced by 2-acetylaminofluorene (2-AAF) and aflatoxin B_1 [AFB_1] (*6*). Several mechanisms have been proposed for the protective effect against mutagenesis *in vitro*. These include the reduction in the formation of the ultimate genotoxic intermediate by interfering with the cytochrome P450-mediated metabolic activation of mutagens and/or the direct interaction of nucleophilic tea components with the genotoxic intermediates, thereby preventing mutagenesis. Aqueous extracts of rooibos and honeybush teas have been shown to possess antioxidant activities *in vitro* (*13, 14*).

Unlike green or black teas, rooibos and honeybush teas contain no caffeine (*11, 15*) and have low tannin content (*11, 16*). The herbal teas also contain phenolic compounds that differ from each other and from green and black teas. Rooibos

flavonoids predominantly consist of dihydrochalcones, flavonols, and flavones. Its flavonoid composition is unique in that it contains aspalathin, which to date has only been isolated from rooibos (*17*), and nothofagin, another rare β-hydroxydihydrochalcone (*18*). On the other hand, honeybush tea contains mainly xanthones, flavanones, and coumestans, with the xanthone, mangiferin, and the flavanone hesperidin constituting the major phenolic compounds (*19-21*). Processing results in a significant decrease in aspalathin and nothofagin in rooibos and mangiferin, isomangiferin, and hesperidin in honeybush (*10*, *18*). At present, very little is known about the oxidized products present in the processed teas. In contrast, green tea mainly contains flavanols, while black tea contains their oxidized products, theaflavins and thearubigins (*22*, *23*).

Although the herbal teas exhibit antimutagenic properties (*6*, *12*) no information is available regarding the possible protective effects against carcinogen metabolism *in vivo*. It is known that the balance between the phase I (carcinogen activating) and the phase II (carcinogen detoxifying) enzymes is critical in the subsequent production of putative carcinogenic or mutagenic metabolites that are ultimately available to interact with the cell. The level and/or activity of these enzymes have been suggested to play an important role in the susceptibility of an individual for developing cancer (*24*). Several compounds that selectively enhance the activity of the phase II enzymes have been identified to impede the production of putative genotoxic metabolites and the formation of preneoplastic lesions and tumors (*25-28*). An increase in phase II detoxifying enzymes in rat liver treated by oltipraz [5-(2-pyrazinyl)-4 methyl-1,2 dithiole-3-thione] has been shown (*29*, *30*) and postulated to be essential for effective chemoprotection against AFB_1-induced hepatocarcinogenesis in rats (*25*). In a clinical trial conducted in residents of Qidong, a high incidence liver cancer area in China, oltipraz was shown to significantly reduce serum albumin DNA adducts of AFB1 (*31*).

The present study investigates the modulation of drug metabolizing enzymes in the liver of rats by rooibos and honeybush teas as compared to green and black teas. The antioxidant status as reflected by the redox state of glutathione and the oxygen radical absorbance capacity (ORAC) in liver of rats exposed to the various tea preparations was also determined.

MATERIALS AND METHODS

Chemicals. The 1-chloro-2,4-dinitrobenzene (CDNB) [97-00-7] and 3,4-dichloro-nitrobenzene (DCNB) [6306-39-4] were purchased from Merck. Reduced [70-18-8] and oxidized [27025-41-8] glutathione were purchased from Roche. BDH laboratories were the suppliers of sodium dithionite [7775-14-6], dimethyl sulfoxide (DMSO) [67-68-5] and ethanol (96%). UDP-glucuronic acid [63700-19-6], *p*-nitrophenol [100-02-7], glutathione reductase, perchloric acid [7601-90-3], 1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate (M2VP), EDTA [60-00-4], and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) [69-78-3] were purchased from Sigma Chemical Co. Phycoerytherin (β-PE) was purchased from ProZyme, and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) [2997-92-4] and Trolox [53188-07-1] were obtained from Aldrich Chemical Co.

Plant Material. Individual batches of processed and unprocessed rooibos and honeybush (*C. intermedia*) teas of the highest quality were a gift from Dr E. Joubert, (ARC Infruitec-Nietvoorbij, South Africa). The black tea (*C. sinensis* var. *assamica*) is a blend of locally produced African tea and Sri Lankan teas (http://www.five-roses.com/about-us/default.htm) and was obtained from a commercial retail outlet in Cape Town, South Africa. The green tea (*C. sinensis* var. *sinensis*) imported from China, was a gift from Vital Health Foods, Kuilsriver, South Africa.

Preparation of Aqueous Tea Extracts. Aqueous extracts were prepared by the addition of freshly boiled tap water to the tea leaves and stems to a concentration of 2 g/100 mL for processed and unprocessed rooibos and black and green teas, while 4 g/100 mL was used for processed and unprocessed honeybush tea. These concentrations are customarily used for tea making purposes (*7, 8*). The mixture was allowed to stand for 30 min at room temperature, filtered (Whatman no. 4), and after cooling, dispensed into water bottles.

Treatment of Animals. Male Fischer 344 rats, 150 g, were obtained from the Primate Unit of the Diabetic Research Group of the Medical Research Council of South Africa. They were individually housed in stainless steel wire-bottomed cages fitted with Perspex houses in a closed environment (24-25 °C), with a 12 h light-dark cycle and 50% humidity. The rats were divided into seven groups consisting of 10 rats per group. The experimental groups each received the various aqueous tea extracts for 10 weeks as their sole source of drinking fluid, while the control group received tap water. Fresh

tea was prepared every second day. The rats were fed *ad libitum* rat cubes (Epol Ltd, Johannesburg, South Africa), and the fluid intake monitored on a daily basis. Body weights were monitored on a weekly basis.

Total Phenolic, Flavonoid Content and Soluble Solid Determinations. The soluble solid content of each tea preparation was determined gravimetrically (sixteen repetitions) during the course of the study after drying 1-mL aliquots at 110 °C for 12 h. The scaled-down Folin-Ciocalteau method with gallic acid as standard was used to determine the total phenolic content (*32*) of the different tea extracts. The phenolic content of the extracts was determined before and after precipitation of the flavonoids to yield the nonflavonoid content, and by difference, the flavonoid content of the tea extracts. Precipitation of the flavonoids was done according to Kramling and Singleton (*33*).

Chemical Pathology. The animals were fasted (16 h) and sacrificed under pentobarbital anesthesia after 10 weeks, and blood was collected from the abdominal aorta. Serum samples were prepared for the determination of clinical biochemical parameters including creatinine, total cholesterol, total iron, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total bilirubin (T.Bili), unconjugated bilirubin (D.Bili), and total protein. Serum analyses were conducted on a Technicon RA 1000 automated analyzer.

Preparation of Microsomal and Cytosolic Liver Fractions. Upon sacrifice, the liver was excised immediately, weighed, frozen in liquid nitrogen, and stored at -80 °C until analyzed. For the preparation of the subcellular fractions, a sample was homogenized in 3 volumes of ice-cold 0.15 M KCl solution for 1 min, using a Thomas homogenizer. The homogenates were filtered through double layer cheesecloth and homogenized with a glass dounce (10 strokes) using a loose pestle. The homogenates were centrifuged at 9000*g* for 10 min, and the cytosolic and microsomal preparations were collected after centrifugation of the supernatant at 100000*g* for 1 h. The microsomes were resuspended in 0.15 M KCl using a glass dounce, centrifuged at 100000*g* for 1 h, resuspended in 0.15 M KCl, and stored with the cytosolic fractions at -80 °C. All procedures were performed at 4 °C. Microsomal and cytosolic proteins were determined by the method of Bradford (*34*) using BSA as standard protein.

Enzyme Assays.

Glutathione S-Transferase Assay. The GST-α activity was measured according to the method of Habig *et al.* (*35*), using CDNB as substrate. The reaction mixture contained 0.1 M potassium phosphate buffer (pH 6.5), 30 mM GSH, and 30 mM CDNB. The reaction was initiated by the addition of cytosol (10 mg protein/ mL), and the increase in absorbance at 340 nm due to the formation of CDNB-GSH conjugates was recorded for 3 min at 25 °C. The specific activity (pmol/min/mg protein) was calculated using a millimolar extinction coefficient of 9.6 for CDNB-GSH. The activity of GST-μ was measured using 60 mM DCNB as substrate (*35*). The reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.5), 86.1 mM GSH, and 60 mM DCNB as substrate cytosolic protein (0.3 mg/mL). Absorbance was measured at 344 nm for 3 min at 25 °C. The specific activity was expressed as pmol/min/mg protein using a millimolar extinction coefficient of 8.5 for the DCNB-GSH conjugate.

UDP-Glucuronosyltransferase Assay. The activity of microsomal UDP-GT was determined spectrophotometrically using *p*-nitrophenol and UDP-glucuronic acid as substrates (*36*). Liver microsomes (1 mg of protein/mL), activated with 0.25% (w/v) Triton X-100, were incubated with 0.1 M Tris-HCl (pH 7.4), 50 mM $MgCl_2$, and 5 mM *p*-nitrophenol for 2 min at 37 °C. The reaction was initiated by addition of UDP-glucuronic acid (30 mM) and was terminated after 10 min by the addition of 5% (w/v) trichloroacetic acid (TCA). After centrifugation (3000*g*), 2 M NaOH was added to the supernatant, and the absorbance was determined at 405 nm. The specific activity was expressed as nmol/min/mg protein using the millimolar extinction coefficient of 18.1.

GSH Analysis. Total glutathione (GSH and GSSG) was measured according to a modified method of Tietze (*37*). Liver cytosolic preparations were treated on ice with 6% (v/v) perchloric acid (PCA) containing freshly prepared 3 mM M2VP and 1 mM EDTA for GSSG and with ice-cold 15% (w/v) TCA for GSH determinations on ice. After centrifugation at 10000*g* for 10 min, 50 μL of the supernatant was added to glutathione reductase (1U) and 75 μM DTNB. The reaction was initiated by addition of 0.25 mM NADPH to a final volume of 200 μL. The change in absorbance was monitored at 410 nm for 5 min, and GSH and GSSG levels were calculated using pure GSH and GSSG as standards.

Oxygen Radical Absorbance Capacity (ORAC). The ORAC assay was based on the procedure described by Cao and Prior (*38*). Free radicals were produced by AAPH, and the oxidation of the fluorescent indicator protein *B*-PE was measured. Both reagents were prepared in 75 mM phosphate buffer (pH 7.0), and 50 μM Trolox was used as standard. The liver samples were homogenized in 4 volumes of the phosphate buffer in a Thomas homogenizer (10 strokes) and centrifuged at 12 000*g* for 10 min at 4 °C. The supernatant was deproteinized using 0.25 M PCA and centrifuged at 16 000*g* for 15 min. The resultant supernatants were then stored at -80 °C prior to analysis. The reaction was performed in 96 well microtiter plates and consisted of 170 μLof *B*-PE (80 μg/mL) and 10 μL of diluted (1:1) sample incubated at 37 °C for 15 min. The reaction was initiated by the addition of 20 μL of AAPH (240 mM), and the fluorescence (emission 590 nm, excitation 530 nm) was recorded every 5 min until reading had declined to less than 5% of initial reading. The ORAC values were calculated and expressed as μM Trolox equivalents/mg wet liver weight.

Statistical Analysis. Data were analyzed by two-way ANOVA using the generalized linear model procedure, and Tukey's Studentized range test was used to determine whether the means differed statistically. When data showed unequal variances, the nonparametric Tukey-type test was used. Values were considered significant if $P < 0.05$. Bonferroni pairwise adjustment was used to compare GSH, GSSG, and UDP-GT values.

RESULTS

Body Weight and Relative Liver Weight Gains. No significant differences ($P > 0.05$) in the daily tea intake between the various groups were noted. No differences were noted in the body weight gain and relative liver weight (percentage of bodyweights) as a result of the tea treatment (Table 1).

Liver and Kidney Function Indicators and Serum Iron and Cholesterol Levels. The effect of the different tea treatments on the liver function enzymes of the experimental animals is summarized in Table 1. No significant differences were noted in the activities of the liver function enzymes AST, ALT, and ALP. The level of total bilirubin, unconjugated bilirubin, and total protein was also not significantly different between the tea treated and control rats (results not shown). Similarly, the levels of creatinine, a

marker for kidney function, total cholesterol, and total plasma iron were not altered.

Soluble Solid, Total Phenolic, Flavonoid Content and Total Phenolic Intake. The soluble solids were significantly higher ($P < 0.001$) in the honeybush extracts than in the rooibos extracts, which is in agreement with the larger amount of tea used for the preparation of the honeybush extracts (Table 2). The soluble solids obtained from the processed tea extracts constituted approximately 50% of the unprocessed herbal tea extracts. The soluble solids of the black tea were significantly higher ($P < 0.05$) when compared to the green, unprocessed rooibos and the processed herbal teas.

The total phenol content of the processed rooibos tea's soluble solids did not differ significantly from its unprocessed counterpart, but processed honeybush tea had a significantly lower ($P < 0.001$) phenol content when compared to the soluble solids of unprocessed honeybush tea. Green and black teas had a similar phenolic content (Table 2). The total phenolic content of unprocessed rooibos and green tea's soluble solids was significantly higher than that of unprocessed honeybush tea. Black tea soluble solids exhibited phenolic levels very similar to those of processed rooibos and were significantly higher than those of the processed honeybush tea. The flavonoid content of the green and black teas was approximately 20 and 30% higher when compared with that of the processed and unprocessed herbal teas, respectively (Table 2). There were no significant differences in the phenolic intake of the rats that received the unprocessed rooibos, green, and black tea, while rats that consumed the unprocessed honeybush tea had the highest phenolic intake ($P < 0.05$). Rats that received the processed herbal teas ingested the lowest amount of total phenols when compared with the unprocessed herbal teas and green and black teas. When considering the flavonoid intake, rats receiving the unprocessed honeybush tea, green, and black teas had a significantly ($P < 0.001$) higher intake when compared to the rats consuming the unprocessed rooibos tea. The flavonoid intake of the rats receiving the processed herbal teas was significantly ($P < 0.001$) less when compared with their processed counterparts.

Table 1 Effect of tea treatments on the body weight gain, relative liver weight, and selected blood clinical chemical parameters related to liver and kidney function[a]

Treatment	Body weight gain (g)	Relative liver weight (g)	AST (U/L)	ALT (U/L)	ALP (U/L)	Creatinine (μmol/L)	Cholesterol (mmol/L)	Total iron (μmol/L)
Control (water)	109.3 ± 17.7a	7.26 ± 0.97a	333.6 ± 68.2a	179.4 ± 64.33a	134.8 ± 10.2a	82 ± 6.7a	1.31 ± 0.19a	15.10 ± 1.54a
Rooibos tea processed	112.0 ± 15a	7.00 ± 0.80a	351.5 ± 55a	123.9 ± 24.5a	144.6 ± 22.4a	88.5 ± 6.5a	1.64 ± 0.19a	16.45 ± 2.65a
Rooibos tea unprocessed	115.4 ± 18.8a	6.96 ± 1.2a	391.8 ± 66.9a	146.8 ± 47.7a	127.7 ± 36.7a	88 ± 6.2a	1.54 ± 0.23a	13.9 ± 1.6a
Honeybush tea processed	117.8 ± 19.3a	6.72 ± 0.96a	320.6 ± 56.7a	145.4 ± 91.6a	133.1 ± 25.3a	86.1 ± 3.5a	1.55 ± 0.28a	13.8 ± 2.1a
Honeybush tea unprocessed	106.1 ± 15.4a	7.53 ± 0.77a	311.7 ± 39.1a	132.1 ± 30.3a	144.5 ± 30.5a	85.7 ± 4.35a	1.58 ± 0.27a	16.75 ± 4.23a
Black tea	127.8 ± 18.2a	6.53 ± 0.65a	356.2 ± 100.7a	182.3 ± 94.3a	132.6 ± 12.9a	76.1 ± 5.22a	1.46 ± 0.25a	14.48 ± 2.19a
Green tea	121.3 ± 10.6a	7.04 ± 0.85a	386.7 ± 63.2a	143.1 ± 67.4a	133.7 ± 18.9a	82.5 ± 7.1a	1.53 ± 0.1a	14.26 ± 2.83a

[a] Values in columns are means ± STD of 10 rats per group. Means followed by the same letter are not significantly different.

Cytosolic Glutathione S-Transferases. Exposure of rats to various aqueous tea extracts did not significantly affect the activity of GST-μ (mu). However, there was a significant increase ($P < 0.001$) in the activity of GST-α (alpha) in the livers of rats fed the South African herbal teas when compared to the control and green and black tea treated groups (Table 3).

Microsomal glucuronosyl transferases. A significant increase ($P < 0.001$) in the UDP-GT activity was evident following treatment of the animals with unprocessed rooibos tea, while unprocessed honeybush tea also marginally ($P = 0.06$) increased the activity of the enzyme. Animals receiving green, black, or processed herbal teas did not significantly alter the activity of the enzyme (Table 3).

Glutathione levels. The concentration of GSH in the livers of the experimental animals was not significantly affected by any of the teas, although the South African herbal teas tended to increase the level (non-significantly) when compared with the control and black and green tea groups (Table 4). A significant decrease ($P < 0.05$) in GSSG levels was noted in the liver of rats exposed to the various tea preparations. Processed and unprocessed rooibos tea also caused a significantly lower ($P < 0.05$) GSSG level in the liver when compared to rats drinking the processed and unprocessed honeybush and green and black teas.

Due to the changes in the GSH and GSSG levels, the GSH/GSSG ratio significantly increased ($P < 0.05$) in the liver of the rats drinking the unprocessed and processed rooibos and unprocessed honeybush teas, while processed honeybush tea marginally ($P = 0.06$) increased the ratio (Table 4). Although green and black teas markedly increased the GSH/GSSG ratio when compared to the control group, this increase was not statistically significant.

Table 2 Different intake parameters of male Fischer 344 rats fed various tea preparations over a period of 10 weeks.

Treatment[$]	Soluble solids (mg/mL)	Total phenolic content (mg Gallic acid equivalents/ mg soluble solids)	Liquid intake/ day/100 g BW (mL)	Total phenolic intake (mg Gallic acid equivalents/ day/100 g BW)*	Flavonoids (% of total phenolic content)**	Total flavonoid intake (mg Quercetin equivalents/ day/100 g BW)
Control (water)	None	None	9.7 ± 1.45a	None		None
Rp tea	2.59 ± 0.44a	0.32 ± 0.07a	7.90 ± 1.01a	6.40 ± 0.08a	63.13 ± 1.69a	2.94 ± 0.89a
Rg tea	5.36 ± 0.81b	0.36 ± 0.06a	8.29 ± 0.74a	16.12 ± 0.18b	76.02 ± 2.29b	11.97 ± 1.76b
Hp tea	5.96 ± 0.78b	0.15 ± 0.05b	8.10 ± 0.85a	7.29 ± 0.19a	63.09 ± 3.09a	3.73 ± 1.04a
Hg tea	11.78 ± 1.18c	0.23 ± 0.04ab	8.58 ± 0.92a	22.90 ± 0.36c	74.86 ± 1.39b	19.07 ± 2.37c
Black tea	8.37 ± 0.66c	0.26 ± 0.03a	8.03 ± 0.52a	17.30 ± 0.11b	92.9 ± 2.35c	15.14 ± 2.09c
Green tea	7.21 ± 0.82b	0.30 ± 0.04a	7.92 ± 0.35a	17.02 ± 0.06b	92.89 ± 0.7c	16.48 ± 1.91c

Parameters of tea intake are mean of 16 determinations. Values in columns are means ± STD of ten rats per group. Means followed by the same letter are not significantly different. If the letters differ then $P<0.001$. [$]Aqueous solutions (2%) were prepared of rooibos processed (Rp), unprocessed (Rg), green and black teas and 4% (w/v) of honeybush processed (Hp) and unprocessed (Hg). *Calculation of the total phenolic intake was based on the soluble solids intake obtained from the average liquid intake/day. **Flavonoids were determined after subtracting the non-flavonoid content from the total phenolic content (Materials and Methods)

Table 3 Effect of unprocessed and processed herbal and green and black teas on the activities of the cytosolic glutathione S-transferases and the microsomal UDP-glucuronosyl transferases in livers of rats exposed to the various teas for 10 weeks.

Treatment	GST-μ (mu) (pmol/min/mg prot)	GST-α (alpha) (pmol/min/mg prot)	UDP-GT (nmol/min/mg prot)
Water	2.41 ± 0.39a	2.39 ± 0.40a	31.55 ± 3.97a
Rooibos processed tea	2.96 ± 0.77a	4.60 ± 1.10b	29.32 ± 3.09a
Rooibos unprocessed tea	3.04 ± 0.64a	4.34 ± 1.03b	45.09 ± 4.49b
Honeybush processed tea	2.50 ± 0.43a	3.87 ± 0.63b	37.7 ± 5.88ab
Honeybush unprocessed tea	2.43 ± 0.74a	3.45 ± 1.13b	40.81 ± 6.56(b)
Black tea	2.15 ± 0.47a	2.39 ± 0.66a	33.09 ± 3.67a
Green tea	1.96 ± 0.38a	2.06 ± 0.46a	24.55 ± 2.57aa

Values in columns are means ± STD of ten rats per group. Means followed by the same letter are not significantly different ($P > 0.05$). If letters differ then $P < 0.05$. Letters in parenthesis then $P < 0.1$. DCNB was used as substrate for GST-mu determination and CDNB.for GST-alpha,

Oxygen Radical Absorbance Capacity (ORAC). The ORAC values in the liver of the rats were significantly ($P < 0.001$) and marginally lowered by the green and black teas, respectively. No effect was noticed on the hepatic ORAC level in the rats treated with the herbal teas (Table 4).

Table 4 Effect of unprocessed and processed herbal and green and black tea on reduced glutathione (GSH), oxidized glutathione (GSSG), the ratio GSH/GSSG and oxidative capacity (ORAC) in livers of rats exposed to the various teas for 10 weeks.

Treatment	GSH (μM/mg protein)	GSSG (μM/mg protein)	GSH:GSSG Ratio	ORAC (μM Trolox equivalents/mg protein)
Water	9.35 ± 2.00a	1.23 ± 0.32a	7.98 ± 2.50a	10.49 ± 1.91a
Rooibos processed tea	15.06 ± 5.38a	0.42 ± 0.13b	38.89 ± 12.19b	11.47 ± 1.11a
Rooibos unprocessed tea	16.39 ± 5.33a	0.40 ± 0.13b	42.1± 9.66b	10.74 ± 1.98a
Honeybush processed tea	13.6 ± 2.67a	0.70 ± 0.16c	20.99 ± 7.01(b)	11.28 ± 1.84a
Honeybush unprocessed tea	18.56 ± 6.2a	0.51 ± 0.18c	43.73 ± 12.73b	10.10 ± 1.20a
Black tea	10.67 ± 2.91a	0.87 ± 0.29c	12.9 ± 4.11a	7.99 ± 0.83b
Green tea	9.2 ± 2.12a	0.76 ± 0.15c	13.3 ± 1.77a	8.72 ± 1.96ab

Values in columns are means ± STD of ten rats per group. Means followed by the same letter do not differ significantly ($P > 0.05$). If letters differ then $P < 0.05$. Letters in parenthesis indicate marginal effects ($P < 0.1$).

DISCUSSION

Certain dietary constituents may influence the incidence of diseases (e.g. cancer) by modulating the enzyme systems responsible for the metabolic activation/detoxifying of chemical carcinogens in the cell (*39*). In this regard, the popularity of herbal teas has increased during the past twenty years (*40*) following advances made in our knowledge on the anticancer properties of green and black teas (*3*, *41*, *42*). Because the phenolic constituents of the two South African herbal teas differ from green and black teas, it is imperative to investigate the possible protective mechanisms of rooibos and honeybush teas against the adverse effects of xenobiotics *in vivo*.

Consumption of aqueous extracts of rooibos and honeybush teas, as sole substitutes for drinking water, did not cause any adverse effects in the liver and kidney of the rats. The total serum iron levels were not altered, indicating that none of the teas, irrespective of the difference in phenolic composition, interfered with iron uptake in the

present study. A study by Hesseling (*43*) showed that rooibos tea did not affect iron absorption in a human population; however, conflicting findings have been reported with respect to the effect of black and green teas on iron uptake in humans (*44*, *45*).

Antioxidant activity has been demonstrated for rooibos (*9*) and honeybush tea extracts (*46*) in various *in vitro* systems (e.g., scavenging of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) and superoxide anion radicals). These studies showed that honeybush tea exhibited a significantly lower antioxidant activity than rooibos tea. This could be attributed not only to the difference in the phenolic profile but also to the lower phenolic content of honeybush soluble solids. In the present study, the low levels were compensated for by the increased amount of tea used in preparation of these extracts. The latter did not negatively impact the consumption and/or the body weight gain and liver function of the rats. The phenolic intake of animals receiving green and black teas was similar or equal to the intake of animals receiving the unprocessed rooibos tea and 3-4 times higher when compared to the processed honeybush and processed rooibos tea. In a comparative study, it was demonstrated that aqueous extracts of green tea exhibit higher antioxidant potency than those of black tea, while unprocessed rooibos is comparable to black tea. Processed rooibos tea extracts exhibit the lowest antioxidant activity (*47*). When using the ORAC assay, the total antioxidant capacity of green and black tea compares favorably to other drinks prepared from fruit and vegetables (*48*). In the present study, however, green and black tea marginally ($P < 0.1$) to significantly ($P < 0.05$) reduced the hepatic ORAC level, respectively, while no effect was noticed with the herbal teas. It is not known whether a relationship exists between the reduced hepatic ORAC and the total phenolic intake of the rats fed the green and black teas under the present experimental conditions.

Reduced glutathione, a powerful intracellular antioxidant that plays an important role in stabilizing many enzymes (*49*), could also be considered as a good marker for the antioxidative capacity in tissue (*50, 51*). Several clinical conditions are associated with a decrease in the GSH level in the cell that may result in a lowered cellular redox potential (*52*). Van Acker *et al.* (*51*) showed that GSH has the ability to regenerate a-tocopherol from its radical, maintaining the levels of α-tocopherol at a level to protect the cell membranes against lipid peroxidation. In this regard, flavonoids mimic the antioxidant activity of R-tocopherol, suggesting an interaction between flavonoids and GSH. Nanjo *et al.* (*53*) showed that the addition of green tea catechins to diets high in

palm and perilla oil prevented a decrease in the plasma α-tocopherol concentration in rats. Rooibos and honeybush teas significantly decreased the level of GSSG in the liver resulting in an increase of the GSH/GSSG ratio, presumably by stabilizing GSH. The level of GSH was markedly (not significantly) higher in the liver of the herbal-tea-treated rats. Green and black teas significantly ($P < 0.05$) reduced GSSG, while the GSH level was not affected. Changes in the GSH and GSSG levels resulted in a significant increase in the GSH/GSSG ratio in the liver of the rats fed the herbal teas, while it was markedly (not significantly) increased with the green and black teas. Sohn *et al.* (*54*) also showed that neither the black nor the green tea affected the liver concentrations of GSH in male F344 rats when treated with 2% aqueous extracts of green and black teas for 6 weeks. The GSH/GSSG ratios were not reported. Despite the differences in the type of flavonoids, the unprocessed herbal teas exhibited a similar effect on the GSH/GSSG ratio while there was an approximate 50% reduction in the case of the processed honeybush tea. The significant increase in the GSH/GSSG ratio in the liver of the herbal-tea-treated rats may be indicative of a reduced oxidative stress or an increased antioxidant capacity in the cell, thereby lowering the susceptibility to oxidative damage. The marked increase obtained with the green and black tea suggests that, in the present study, the phenolic components of the herbal teas were more effective in increasing the antioxidant status in the liver.

Stabilization of GSH in the liver may also result in an increase in the endogenous detoxification capacity, as glutathione is known to, either directly or via the glutathione S-transferases, interact with reactive genotoxic metabolites, thereby reducing the likelihood of damage to cellular DNA (*28, 55*). With respect to metabolism, the carcinogenicity of compounds (e.g. the mycotoxin AFB_1 and 2-AAF) is modulated by the interaction with the phase I activating or the phase II detoxifying enzymes (*27*, *28*, *56*, *57*). Studies by Siess *et al.* (*28*) in Wistar rats suggest that dietary flavonoids inhibit AFB_1 carcinogenesis by decreasing the covalent binding of AFB_1 to liver DNA as a result of the production of conjugated AFB_1 metabolites via the induction of phase II enzymes GST-α and UDP-GT. Rats treated with coumarin, a natural benzopyrone, were protected from developing hepatic AFB_1-induced preneoplastic lesions (*27*). The induction of class GST-A5, a subunit of GST-α, was implied to play an important protective role. In the present study, the activity of GST-α was increased in the livers of rats fed the processed and unprocessed rooibos and honeybush teas. The black and

green teas showed no effect when compared to the control group, which is in accordance with studies conducted by Bu-Abbas *et al.* (58) and Sohn *et al.* (*54*) using male Wistar and Fischer rats, respectively. However, an increase in the activities of GST-α and GST-μ when using aqueous extracts of black tea in human Chang liver cells has been reported (*1*). Another study also showed a significant induction of GST by green tea leaves when fed to Wistar rats at various concentrations between 4 and 63 weeks (*59*).

Similar discrepancies seem to exist with respect to the induction of UDP-GT by green and black teas. Under the present experimental conditions, no induction of UDP-GT activity was found in male Fischer rats treated with 2% tea in their drinking water for 10 weeks. The induction of UDP-GT activity in the microsomes has been suggested as a major mechanism of tea as a chemopreventive agent (*60*). Unprocessed rooibos tea significantly increased the activity of the microsomal UDP-GT in the liver, while unprocessed honeybush tea only showed a marginal significant ($P < 0.1$) increase. The increase in the glucuronidation capacity was suggested to facilitate the metabolism of chemical carcinogens into inactive, readily excretable products, thereby reducing their possible interaction with cellular DNA (*58*). The lack of induction of UDP-GT activity in processed rooibos and honeybush tea could be due to the change in phenolic composition during processing of these teas (9, *10*, *21*). The present study showed no difference in the induction of the activity of the enzyme by the green and black teas. In contrast, several studies indicated that aqueous extracts of black tea and to a lesser extent green tea induced the activity of UDP-GT using *p*-nitrophenol and 2-aminophenol as substrates for the enzyme (*54*, *56*, *60*, *61*). However, when using 4-methyl-umbelliferone as a substrate none of the teas increased the activity of the enzyme. Decaffeinated black tea also failed to enhance the activity of the enzyme.

It would appear that many variables exist that could determine the induction of the activity of phase II enzymes by green and black tea. These include the rat species used (*58*), the amount of tea used, duration of treatment and route of administration (*59*), and the substrate used in the enzyme assay (*58*, *61*). Some of these parameters could have contributed to the apparent lack of the induction of enzyme activity by green and black tea reported in the present study.

A preliminary report by Marnewick *et al.* (*62*) indicated that the mutagenicity of

AFB_1 and 2-AAF decreased with the addition of liver cytosolic fractions from male Fischer rats that consumed the South African herbal teas for 10 weeks when compared with controls given water. The present study indicates that enhanced activities of the important phase II enzymes, GST-α and UDP-GT, as well as the increased oxidative status in the liver, may account for this protective effect in the mutagenicity assay. Apart from the induction of phase II enzymes by rooibos and honeybush teas, other mechanisms of antimutagenicity may play a role. Nucleophilic tea components may also directly interact with the genotoxic reactive intermediates of mutagens to reduce their mutagenic potential. However, this may vary with the type of mutagen and needs further investigation (*6*, *63*). Because the phenolic constituents of the two South African herbal teas differ from those of green and black teas, the mechanisms involved in protection against DNA damage could also differ. The induction of phase II hepatic drug metabolizing enzymes and the increased antioxidant status of the liver by these herbal teas may represent a promising tool for chemoprevention against cancer in humans as they are consumed on a regular basis locally and are becoming increasingly important as a nutraceutical product globally.

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CHAPTER 5

Ex vivo Modulation of Chemical-induced Mutagenesis by Subcellular Liver Fractions of Rats Treated with Rooibos (*Aspalathus linearis*) Tea, Honeybush (*Cyclopia intermedia*) Tea, as well as Green and Black (*Camellia sinensis*) Teas

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ABSTRACT

Male Fischer rats were given unprocessed (not oxidized) and processed (oxidized) rooibos and honeybush teas as well as green and black teas as a sole source of drinking fluid for 10 weeks, and sub cellular liver fractions were prepared. Cytosolic fractions of rats consuming the unprocessed herbal teas, green and black teas significantly ($P < 0.05$) protected against 2-acetylaminofluorene (2-AAF)-induced mutagenesis in the *Salmonella* mutagenicity test with strain TA 98, using Aroclor 1254-induced microsomes. A marginal or no protection was obtained with the processed herbal teas. The mutagenic response of aflatoxin B_1 (AFB_1) against *Salmonella* strain TA 100 was significantly ($P < 0.05$) inhibited by cytosolic fractions from rats treated with processed and unprocessed herbal teas, while no effect was obtained with the green and black teas. Microsomal fractions prepared from livers of rats treated with both the processed and unprocessed rooibos teas and the unprocessed honeybush tea, significantly ($P < 0.05$) reduced the activation of AFB_1 while no protection was observed against 2-AAF-induced mutagenesis. In contrast, microsomal fractions from rats treated with the green, black and unprocessed honeybush teas significantly ($P < 0.05$) enhanced the mutagenic response of 2-AAF. None of the tea treatments significantly affected the concentration of the microsomal liver cytochrome P450.

KEYWORDS: Aflatoxin B_1; 2-Acetylaminofluorene; Mutagenesis; *Ex vivo* protection; Rooibos; Honeybush

INTRODUCTION

Food borne mutagens and carcinogens are readily converted into reactive genotoxic intermediates by phase I metabolizing enzymes. Following this metabolic step, the detoxification pathways convert the potential genotoxins to less reactive metabolites, which can be more readily excreted [1]. Studies in experimental animals revealed that many food-derived and/or synthetic components inhibit the carcinogenic response of food contaminants e.g. 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), a heterocyclic amine produced during the cooking of protein-rich food, benzo[a]pyrene (B[a]P), a polycyclic aromatic hydrocarbon from heating food materials and aflatoxin B_1 (AFB_1), a mycotoxin produced by *Aspergillus flavus* [2,3]. Dietary constituents can selectively modulate the drug-metabolizing enzymes thereby altering the metabolic fate of the carcinogen in the cell [4–6]. This could lead to either a decrease or in some cases even an increase in the level of the active carcinogenic metabolite(s) available to interact with DNA, which is known to be an important determinant in the development of cancer.

Epidemiological studies indicated that the frequent intake of certain food components plays an important role in reducing the risk for cancer development in humans [7–10]. With growing scientific evidence of the beneficial health properties of green and black teas [11–13] the health-promoting potential of two unique South African herbal teas, rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*) are of interest. Rooibos and honeybush teas have been used as herbal beverages and to a lesser extent as herbal 'medicines' in South Africa before the 1800s [14]. The popularity of these teas can be ascribed to the low tannin content [15,16] and the absence of caffeine [15,17]. The established pharmacological effects of rooibos tea and to a certain extent honey-bush tea include anti-oxidative [18–20], anti-aging [21], anti-viral and anti-inflammatory [22] properties, and anti-mutagenic activity in test systems *in vitro* [23,24]. Most of the protective effects against mutagenesis of green and black teas were ascribed to the polyphenolic compounds, the catechins. Rooibos and honeybush teas contain a wide variety of phenolic compounds that not only differs from that of the green and black teas but also from one another. Rooibos tea predominantly contains dihydrochalcones, flavonols and flavones [25,26] and honeybush tea (*C. intermedia*) contains xanthones, flavanones, and coumestans [27,28]. In contrast, green tea mainly contains flavanols while black tea contains the oxidized polymeric compounds, the theaflavins and thearubigins [29,30].

Many studies focused on the inhibition of mutagenesis by tea and tea polyphenols *in vitro*, but these modulating properties have not been fully elucidated *in vivo* [31]. Considering the potent anti-mutagenic activity of rooibos and honeybush teas *in vitro* [18,24] very little information is available whether similar protective mechanisms exist *in vivo* and what type of mechanism is involved. A study by Sasaki *et al.* [23] in hamster ovary cells and male ICR mice showed that extracts of processed rooibos tea decreased the number of chromosome aberrations and micronucleated reticulocytes, respectively, after exposure to benzo[*a*]pyrene (B[a]P) and mitomycin C (MMC). The present study investigated the *ex vivo* modulating effects of sub-cellular hepatic fractions of rats treated with processed and unprocessed herbal teas as compared with green and black teas against 2-acetylaminofluorene (2-AAF)-and AFB_1-induced mutagenesis in the *Salmonella* mutagenicity assay.

MATERIALS AND METHODS

Chemicals. 2-AAF [53-96-3] and AFB_1 [1162-65-8], biotin [58-85-5], histidine [71-00-1], nicotinamide adenine dinucleotide phosphate (NADP) [1184-16-3], glucose-6-phosphate (G-6-P) [54010-71-8] and the enzyme, glucose-6-phosphate dehydrogenase (G-6-PDH) [9001-40-5] were purchased from Sigma (SA). Dimethyl sulfoxide (DMSO) [67-68-5] was obtained from BDH Laboratory Supplies (Poole, UK). Agar and Nutrient Broth No. 2 were purchased from the Difco Laboratories (Detroit, USA) and Oxoid (Hampshire, UK), respectively. All other solvents used were of analytical grade.

Tea preparations. Aqueous extracts of processed and unprocessed rooibos and honeybush and of green and black teas were prepared by the addition of freshly boiled tap water to the tea leaves and stems (2 g/100 ml) for processed and unprocessed rooibos, and black and green teas and 4 g/100 ml for processed and unprocessed honeybush tea. The herbal tea concentrations used are customary for tea making purposes [32,33] in South Africa. The mixture was allowed to stand for 30 min at room temperature, filtered (Whatman no. 4) and after cooling, dispensed into water bottles. Individual batches of processed and unprocessed rooibos and honeybush teas of the highest quality were supplied by Dr. E. Joubert. The black tea (*Camellia sinensis* var. *assamica*) was a blend of locally produced African tea and Sri Lankan teas (http://www.five-roses.com/about-us/default.htm) and was obtained from a commercial retail outlet in Cape Town, South Africa. The green tea (*C. sinensis* var. *sinensis*), imported from China, was a gift from Vital

Health Foods, Kuilsriver, South Africa.

Treatment of animals. Seventy male Fischer 344 rats, weighing 150–190 g, were obtained from the Primate Unit (Diabetic Research Group of the Medical Research Council of South Africa), randomly divided into seven treatment groups consisting of ten rats per group and housed individually in stainless steel wire-bottomed cages fitted with perspex houses in a closed environment (24–25 ^{0}C), with a 12 h light–dark cycle and 50% humidity. Rats had free access to the various aqueous tea extracts for 10 weeks as their sole source of drinking fluid while the control group received tap water. The rats were fed rat cubes (Epol Ltd., Johannesburg, South Africa) *ad libitum* and the fluid intake monitored on a daily basis while the tea was freshly prepared every second day. The general condition of the rats was monitored daily throughout the experiment while the body weights were determined on a weekly basis.

Preparation of microsomal and cytosolic liver fractions. The animals were sacrificed under pentobarbital anesthesia and blood was collected from the abdominal aorta and subjected to clinical chemical analyses [34]. Livers were immediately excised, weighed and a sub-sample was homogenized in three volumes of ice-cold 0.15 M KCl for 1 min using a Thomas homogenizer. The homogenates were filtered through double-layer cheesecloth, and homogenized in a glass tissue-grinder (10 strokes) using a loose plunger. The homogenates were centrifuged at 9000*g* for 10 min and the cytosolic and microsomal preparations collected after centrifugation of the resultant 'S-9' supernatant at 100,000*g* for 1 h. The microsomes were collected in 0.15 M KCl, homogenized in a glass tissue grinder using a loose plunger, centrifuged at 100,000*g* for 1 h, resuspended in 0.15 M KCl and stored with the cytosolic fractions at −80 ^{0}C. All procedures were performed under sterile conditions at 4 ^{0}C. Microsomal and cytosolic proteins were determined by the method of Bradford using bovine serum albumin as protein standard [35].

The S-9 homogenate used in the standard *Salmonella* mutagenicity assay was prepared from Aroclor 1254-induced rats as described previously [36], aliquoted in sterile vials and stored at −80 ^{0}C. For the preparation of Aroclor-induced microsomes the resultant S-9 homogenate was fractionated as described above.

Cytochrome P450 determination. The cytochrome P450 content of the S-9 homogenates and microsomal fractions was determined from the dithionite-reduced

difference spectrum of carbon monoxide-saturated samples and expressed as nmol/mg protein using a millimolar extinction coefficient of 91 [37].

***Salmonella* mutagenicity /anti-mutagenicity assay.** The *Salmonella* mutagenicity assay was conducted according to the method described by Maron and Ames [36] with minor modifications. Two known mutagens, 2-AAF and AFB_1, requiring metabolic activation, were used against tester strains TA 98 and TA 100, respectively. The tester strains were obtained from Dr. B.N. Ames (Berkeley, California, USA). Stock solutions of the different carcinogens were freshly prepared on the day of the experiment using dimethyl sulfoxide as solvent. The mutagenic response of 2-AAF and AFB_1 in the standard assay was conducted using S-9 fractions from Aroclor-1254 treated rats at a concentration of 2 mg protein/ml S-9 mixture and cytochrome P450 (CYP450) at 0.6 nmol/mg protein. Five replicate plates were included for each sample.

Cytosolic modulation assay. For the cytosolic modulation assay, the S-9 activation mixture consisted of phosphate buffer (0.1 M) pH 7.4, salt solution (0.03 M KCl; 8 mM $MgCl_2$), glucose-6-phosphate (5.3 mM), NADP (0.03 M) and Aroclor-induced microsomes (1 mg protein/ml; 2.8 nmol/mg protein). The optimum cytosolic concentration to compare the protective properties of the different cytosolic preparations was established first. The various cytosolic preparations from the herbal tea-and water-treated rats (one rat per group) were tested at two protein concentrations (0.25 and 1 mg protein/ml S-9 mixture) against strain TA 98, with 2-AAF as a mutagen. Subsequent experiments were conducted using the different cytosolic preparations at a protein level of 0.25 mg/ml S-9 mixture, with 2-AAF (5 μg/plate) and AFB_1 (15 ng/plate) as mutagens. The total protein concentration (1.25 mg/ml) of the S-9 mixture used in the comparative study was below that proposed for mutagenicity testing (1.6 mg protein/ml S-9 mixture) of potentially mutagenic compounds when utilizing the plate-incorporation assay [36]. This would have excluded the possible occurrence of certain artifacts induced by the different cytotosolic fractions such as bacterial cytotoxicity and/or the inhibition of mutagenesis due to the high level of protein added.

Microsomal activation assay. The mutagen-activating potential of microsomes isolated from tea-treated rats was determined using the standard plate-incorporation assay

[36]. The activation mixture consisted of 0.1 M phosphate buffer (pH 7.4), salt solution (0.03 M KCl; 8 mM MgCl2), glucose-6-phosphate (5.3 mM), NADP (0.04 M) and glucose-6-phosphate dehydrogenase (equivalent to 2 U/ml). The microsomes of the different tea-treated rats were incorporated at a level of 1 mg protein/ml in the activation mixture, while the mutagen concentrations used were 100 mg/plate for 2-AAF and 50 ng/plate for AFB_1.

Control plates, containing only DMSO (used as solvent vehicle) were also included to obtain spontaneous revertant counts. All plates were incubated at 37 ^{0}C for 48 h and the histidine revertants were counted using a Quebec Colony Counter (America Optical Corp., Buffalo, New York). All experiments were repeated twice and five replicates for each sample were included.

Statistical analysis. Analysis of variance (ANOVA) was performed using the statistical analysis system (SAS) programme. Data showing equal variances were analyzed using the parametric Tukey *T*-test. When data showed unequal variances (Kruskal–Wallis test) the non-parametric Tukey-type test was used.

RESULTS

Tea intake profiles and effect on rat body weight parameters. The various tea treatments had no adverse effects on the body weight gain, relative liver weight, blood clinical chemical changes and daily fluid intake of the rats [34]. Tea intake profiles varied between 8 and 9 ml/100 g body weight per day with no significant difference between the various groups. The total flavonoid intake was significantly higher ($P < 0.001$) in the black, green and unprocessed honeybush tea-treated rats as compared with the rats consuming the processed and unprocessed rooibos and unprocessed honeybush teas (Table 1).

Cytosolic modulation assay

Pilot study. An increase in the mutagenic response of 2-AAF against strain TA 98 with Aroclor 1254-induced microsomes was noticed when the level of the cytosolic fraction from the control rats was increased from 0.25 to 1 mg protein/ml of the S-9 mixture (equivalent to 0.125 and 0.5 mg/plate) (Fig. 1). Significant ($P < 0.05$) protection was obtained with the different cytosolic fractions from the herbal tea-treated rats at concentrations of 0.25 mg/ml of the S-9 mixture. A similar inhibition pattern was obtained with the higher cytosolic content (1 mg/ml S-9) although a weaker protection was noticed with

the processed and unprocessed honeybush teas. Based on these data a cytosolic level of 0.25 mg/ml of the S-9 mixture was used in the study for the different cytosolic fractions of the tea-treated rats, including those given the green and black teas. For comparative purposes, the subsequent studies with AFB_1 as a mutagen against strain TA 100, was also conducted at the same cytosolic protein concentration.

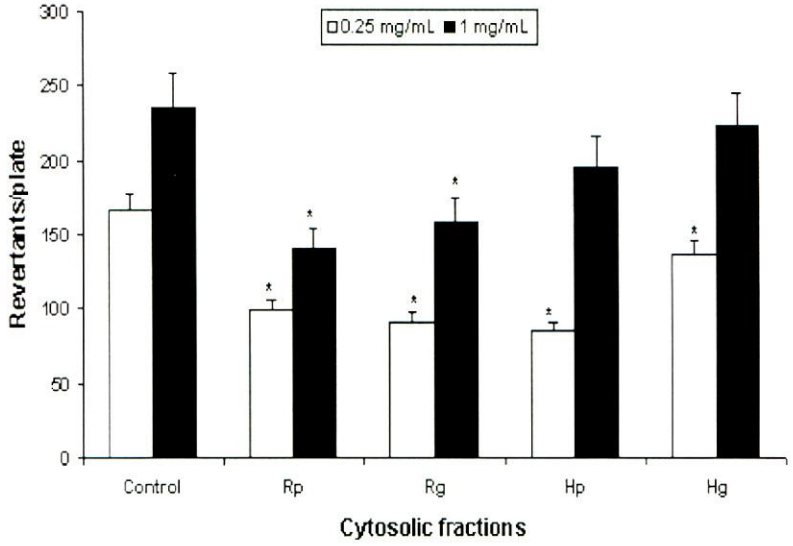

Fig. 1. *Ex vivo* protective effect of different cytosolic protein concentrations (0.25 and 1 mg/ml) on the mutagenicity induced by 2-AAF in *S. typhimurium* TA 98. Rp: processed rooibos, Rg: unprocessed rooibos, Hp: processed honeybush and Hg: unprocessed honeybush. Each value represents the mean and standard deviation (n= 5). * $P < 0.05$ when different cytosolic concentrations were compared with the control. Microsomes (1 mg/ml activating mixture) of Aroclor-induced rats were used to induce metabolic activation of 2-AAF.

Tea-derived cytosolic liver fractions. The protective effects of the different cytosolic preparations obtained from liver homogenates of the tea and the water treated rats against 2-AAF- and AFB_1–induced mutagenesis are summarised in Table 1. When 2-AAF was used as mutagen, only the cytosolic fractions of the rats consuming the unprocessed herbal teas, and the green and black teas significantly ($P < 0.001$) decreased the mutagenicity as compared with the cytosolic fractions prepared from the water-treated control rats. The cytosolic fraction of the rats receiving the processed honeybush tea showed a marginal ($P = 0.053$) protective effect. When AFB_1 was used as mutagen, the cytosolic fractions from the rats treated with both processed and unprocessed rooibos and honeybush teas resulted in a significant ($P < 0.001$) protective effect as compared to the control. No significant protective effect was noted with the cytosolic fractions of the rats given the green and black teas at the specific concentration tested.

Microsomal activation assay. None of the microsomal preparations of the tea-treated rats caused a decrease in the mutagenicity of 2-AAF when compared to the control (Table 2). However, a significant ($P < 0.05$) increase in the number of histidine revertants was obtained with the liver microsomal fractions of the rats treated with the unprocessed honeybush, black and green teas. A marked but not significant increase in the number of histidine revertants was noticed with the microsomal fractions obtained from the rats treated with the processed honeybush tea and the processed and unprocessed rooibos teas.

When AFB_1 was used as mutagen, the microsomal preparations of the rats treated with processed and unprocessed rooibos and unprocessed honeybush teas showed a significant ($P < 0.05$) decrease in the number of histidine revertants. No effect was obtained with the microsomal fractions of the rats treated with the green or black teas, or with the processed honeybush teas. The concentration of microsomal cytochrome P450 (nmol/mg protein) of the different microsomal preparations did not differ significantly as a result of the different tea treatments (Table 2). However, the levels tended to be higher (not significantly) in the microsomes prepared from rats that had received the unprocessed honeybush tea and the black tea.

Spontaneous revertant counts. The spontaneous revertant counts of strains TA 98 and TA 100 were in the range (30-50 and 120-200, respectively) of published values [36].

DISCUSSION

Several mechanisms for the modulating role of black and green tea extracts or purified phenolic compounds on the *in vitro* mutagenic response against various metabolically activated mutagens have been suggested. These include the inhibition of CYP450-dependent bio-activation of the pro-mutagens and/or a prevention of DNA damage by the interaction of tea components with the ultimate carcinogens [12,38-42]. With respect to rooibos and honeybush teas, similar *in vitro* protective mechanisms against 2-AAF-and AFB_1-induced mutagenesis in the *Salmonella* mutagenicity assay were suggested previously [24]. In that study, the anti-mutagenic activity of the aqueous toxic effect to the *Salmonella* bacteria used.

Table 1 Protective effects of liver cytosolic fractions isolated from rats consuming processed and unprocessed rooibos and honeybush teas, and green and black teas against mutagenicity of 2-AAF and AFB_1 in the *Salmonella* assay

Tea treatments	Total flavonoid intake (mg gallic acid equivalents per day/100 g BW)[a]	Revertants per plate	
		TA 98 2-AAF (5 μg/plate)	TA 100 AFB_1 (10 ng/plate)
Control (tap water)	–	220.3 ± 32.5 a	385.0 ± 42.0 a
Processed rooibos tea	2.94 ± 0.89 a	206.7 ± 36.0 a	304.9 ± 41.4 b
Unprocessed rooibos tea	11.97 ± 1.76 b	151.2 ± 30.1 b	303.3 ± 32.8 b
Processed honeybush tea	3.73 ± 1.04 a	191.1 ± 37.6 (b)	306.1 ± 43.9 b
Unprocessed honeybush tea	19.07 ± 2.37 c	161.3 ± 30.3 b	303.2 ± 45.1 b
Black tea	15.14 ± 2.09 c	116.2 ± 27.3 b	359.8 ± 34.8 a
Green tea	16.48 ± 1.91 c	111.6 ± 25.8 b	345.8 ± 50.0 a
Spontaneous revertants	–	30.3 ± 3.7	131.5 ± 11.9
Positive control[b]	–	487.7 ± 74.3	519.7 ± 31.2

Values in columns are means ± S.D.of 10 rats per group. Means followed by the same letter do not differ significantly. If letters differ then $P < 0.05$. Letter in parentheses indicates a marginal effect ($P < 0.1$). Cytosolic preparations from different tea-treated and water-treated rats were incorporated at 0.25 mg/ml activation mixture. Aroclor-activated microsomes (1 mg/ml) were added to the activation mixture. [a]Data from Marnewick et al. [34]. [b]A S-9 preparation (2 mg protein/ml S-9 mix) from Aroclor-induced rats was used.

Table 2 Activation potential of hepatic microsomal fractions isolated from rats consuming the different teas on 2-AAF-and AFB_1-induced mutagenesis

Tea treatments	CYP450 (nmol/mg protein)	Revertants per plate	
		TA 98 2-AAF (100 μg/plate)	TA 100 AFB_1 (50 ng/plate)
Control (tap water)	0.35 ± 0.14 a	351.9 ± 80.0 a	1115.4 ± 167.1 a
Processed rooibos tea (2%)	0.31 ± 0.06 a	527.0 ± 197.5 a	878.4 ± 178.0 b
Unprocessed rooibos tea (2%)	0.38 ± 0.04 a	536.3 ± 114.0 a	907.9 ± 91.1 b
Processed honeybush tea (4%)	0.31 ± 0.06 a	492.1 ± 101.0 a	1102.7 ± 209.5 a
Unprocessed honeybush tea (4%)	0.45 ± 0.11 a	617.7 ± 75.9 b	941.2 ± 154.3 b
Black tea (2%)	0.41 ± 0.09 a	803.8 ± 132.0 c	1150.7 ± 244.3 a
Green tea (2%)	0.37 ± 0.03 a	818.0 ± 149.7 c	1375.2 ± 350.6 a

Values in columns are means ± S.D. of 10 rats per group. Means followed by the same letter do not differ significantly. If letters differ then $P < 0.05$. Microsomes from different tea-treated and control rats were incorporated at a level of 1 mg/ml activation mixture. unprocessed rooibos and honeybush teas resulted in a significant ($P < 0.001$) protective effect as compared to the control. No significant protective effect was noted with the cytosolic fractions of the rats given the green and black teas at the specific concentration tested.

The present study was conducted to establish whether these protective mechanisms would also prevail *in vivo* by monitoring the *ex vivo* anti-mutagenic activity of the cytosolic fractions of tea-treated rats. Rats exposed to the various tea preparations (2 and 4% in their drinking-water) exhibited no hepatotoxic effects when monitoring the serum levels of enzymes indicative of liver function [34]. No cytotoxic effects were observed when incorporating the different cytosolic fractions (0.25 mg protein/ml S-9 activation mixture) in the presence of Aroclor-induced microsomes (1 mg protein/ml S-9 activation mixture). In this regard, no reduction in the spontaneous mutation rate or any cytotoxic effects to the tester strains were monitored when using the Aroclor-induced S-9 containing a higher protein content (2 mg protein/ml S-9 mixture).

When considering the metabolic fate of AFB_1 and 2-AAF, chemopreventive agents have been characterised that selectively reduce their carcinogenic effects. With respect to AFB_1, studies focused on the fate of the reactive mutagenic metabolite, the AFB_1-8,9-epoxide, by either inhibiting its CYP450-dependent activation and/or enhancing its detoxification via conjugation with glutathione (GSH) catalyzed by the cytosolic glutathione-*S*-transferases (GSTs) [43–45]. With respect to the aromatic amines (2-AF, 2-AAF), two different mechanisms exist in the production of the active genotoxic metabolites, one involving the rat liver microsomes and the other both the microsomal and cytosolic cellular compartments [46,47]. In both cases the initial step involves N-hydroxylation with the subsequent addition of either an acetyl group by the microsomal N,O-acetyltransferases or a sulfate via the sulfotransferases in the cytosol [48]. Modulation of the formation of active mutagenic metabolite(s) derived from these aromatic amines could therefore occur at different levels involving both the cytosolic and microsomal compartments [1].

In the present study, liver cytosolic fractions from rats that had received processed and unprocessed rooibos and honeybush tea protected against AFB_1-induced mutagenicity *ex vivo*, while green and black tea showed no cytosolic protection. Unprocessed herbal teas, and green and black teas showed a significant and processed honeybush tea a marginal ($P < 0.053$) cytosolic protective effect against 2-AAF-induced mutagenesis, while processed rooibos tea showed no protection. It is not known whether a protective effect could have been obtained at higher cytosolic levels (>0.25 mg protein/ml S-9 fraction). However, in the case of 2-AAF a lower protective effect was observed at the higher cytosolic concentration (1 mg/ml) in the pilot study. In this regard the presence of

the cytosolic sulfotransferases would have increased the mutagenic response of 2-AAF and, as discussed above, could have decreased the protective effect of the cytosol.

Microsomal fractions obtained from rats treated with the herbal teas, except processed honeybush tea, also showed a decreased metabolic conversion of AFB_1 to its reactive mutagenic intermediate, while the processed honeybush tea and the green and black teas did not significantly alter the activation of AFB_1. In contrast to AFB_1 mutagenesis, none of the herbal, green and black teas decreased the microsomal activation of 2-AAF. Instead, 2-AAF mutagenesis was increased markedly to significantly when utilizing microsomal preparations of the different teas.

The present study provides evidence that active components involved in the *ex vivo* modulation of 2-AAF and AFB_1 metabolism are absorbed from the gut to exhibit their protective effect. Therefore, oral administration of rooibos and honeybush teas is likely to reduce the liver microsomal activation of AFB_1 significantly, while cytosolic component(s) would further reduce the availability of the active mutagenic metabolite to interact with DNA. In contrast, only the cytosolic components are likely to protect against 2-AAF-induced effects and the balance between enhanced microsomal activation versus cytosolic protection could determine the biological outcome *in vivo*.

Differences in the *ex vivo* modulation of mutagenesis induced by 2-AAF could be ascribed, as mentioned above, to differences in the metabolic pathways resulting in the formation of the active metabolite. Whether induction of the microsomal N,O-acetyltransferases could also contribute to enhanced activation of 2-AAF is not known at present.

The exact mechanisms involved in the *ex vivo* protection of the different tea preparations against AFB_1-and 2-AAF-induced mutagenesis are presently unknown. However, a recent study by Marnewick *et al.* [34] indicated that the herbal teas significantly enhance the activity of the microsomal UDP-glucuronosyl transferases, and the cytosolic glutathione-*S*-transferase alpha (GST-α), which could be important in explaining the protective effects of the different sub-cellular liver fractions. The herbal teas also stabilize the level of reduced glutathione (GSH) that may result in an increased antioxidant capacity in the cell. In the present study these parameters could play an important role in the *ex vivo* protection of the cytosolic fractions against AFB_1 and 2-AAF-induced mutagenesis. The protective activity of green and black teas against 2-AAF appears not to proceed the

modulation of GST-α or UDP-GT as they failed to induce the activity of these enzymes under the present conditions [34]. In this regard the stabilization of GSH by the green and black tea seems to be involved. It is not known whether the activity of the sulfotransferases is modulated by green and black teas, thereby reducing the cytosolic activation component of 2-AAF. However, other cytosolic components such as the tea phenols could also be involved in the direct scavenging of reactive AFB_1 and 2-AAF metabolites under the present experimental conditions.

Differences in the microsomal conversion of AFB_1 and 2-AAF could be related either to the induction of specific CYP450 isoforms involved in their metabolism or to direct metabolic competition between the flavonoids and the carcinogens. Various flavonoids are metabolized by certain CYP450 isoforms, e.g. CYP1A2, and they may competitively reduce the CYP1A2-mediated activation of AFB_1, thereby inhibiting its mutagenic activity [4]. Different flavonoids have also been shown to selectively induce the activity of enzymes of the CYP family [49] e.g. the flavanones induce CYP2B1/2 and the flavones CYP1A and 2B isozymes [50]. Differences in the induction pattern of these isozymes could therefore play an important role in the metabolic fate of a potential mutagen or carcinogen *in vivo*. Treatment of rats with green and black tea from 4 to 6 weeks significantly induced different isoforms of CYP450 including CYP1A1, 1A2, 2B, 4A1 [51–53]. In the present study the total daily flavonoid intake was shown to be significantly ($P < 0.05$) higher in rats fed the black, green and unprocessed honeybush teas, suggesting that the induction of the CYP450 isoforms could have enhanced 2-AAF metabolism yielding the active mutagenic metabolite. With respect to the protective effect of the herbal teas against AFB_1 metabolism it would appear that the phenolic constituents of the herbal teas selectively induce different isoforms of CYP450 that could direct the metabolism away from the formation of the active mutagenic metabolite.

Various phenolic tea constituents exhibit different protective mechanisms *in vitro* depending on the chemical nature and metabolic fate of the carcinogen [12]. Variation in the protective effects of the herbal, green and black teas might also be ascribed to the difference in phenolic constituents and their selective effects on the drug-metabolizing enzymes. The present study confirms that the *in vitro* anti-mutagenic activity of the South African herbal teas against AFB_1 and 2-AAF-induced mutagenesis is likely to be active under *in vivo* conditions as well, although the mechanisms may differ. This is the first report on the *ex vivo* protective effects of rooibos and honeybush teas and it provides

valuable information regarding their possible health-promoting properties.

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CHAPTER 6

Chemoprotective Properties of Rooibos (*Aspalathus linearis*), Honeybush (*Cyclopia intermedia*), Green and Black (*Camellia sinensis*) Teas Against Cancer Promotion Induced by Fumonisin B_1 in Rat Liver

For submission to Carcinogenesis

Chemoprotective properties of rooibos (*Aspalathus linearis*), honeybush (*Cyclopia intermedia*), green and black (*Camellia sinensis*) teas against cancer promotion induced by fumonisin B_1 in rat liver

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Abstract

A rat liver carcinogenesis model using diethylnitrosamine (DEN) as initiator was utilized to investigate the chemoprotective properties of unprocessed and processed rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*) herbal teas and green and black teas against fumonisin B_1 (FB_1) promotion. The mean FB_1 and tea intake profiles over the three week promotion treatment were similar between the different groups, although weekly differences were noted. The total body weight gain of the rats consuming the various teas did not differ from the DEN-FB group, but inter-group differences among the tea treated-rats were noted. Consumption of processed and unprocessed rooibos teas variably decreased the relative liver weights when compared to the DEN-FB_1 control and other tea treated rats. Unprocessed rooibos tea significantly ($P < 0.05$) and, to some extent, unprocessed honeybush increased FB_1-induced liver function enzymes. The honeybush and black teas decreased the FB_1-induced elevation of serum creatinine while the honeybush teas as well as green tea decreased serum cholesterol. Green tea also significantly ($P < 0.05$) further reduced the oxygen radical absorbance capacity (ORAC) effected by FB_1 in the liver while unprocessed rooibos, processed rooibos and processed honeybush exhibited a stabilizing effect. The processed herbal teas and unprocessed honeybush tea significantly ($P < 0.05$) decreased TBARS levels while the unprocessed rooibos, black and green teas showed

no effect. Although the total iron levels in the serum were not altered by the various teas when compared to the DEN-FB_1 control, both honeybush teas significantly ($P < 0.05$) and green tea marginally ($P < 0.1$) increased the levels when compared to the rooibos teas. Unprocessed rooibos tea significantly ($P < 0.05$) decreased the hepatic reduced glutathione (GSH) to oxidized glutathione (GSSG) ratio due to a decreased level of GSH. The decrease GSH:GSSG ratio obtained with green and black tea was related to an enhanced GSSG level suggesting an increased hepatic stress. All the tea extracts significantly ($P < 0.05$) increased the relative amount of mini foci (5-10 µm) when expressed as a percentage of the total, whilst reducing the relative focal size of 11-20 µm, thereby delaying focal development. The relative amount of the larger foci was significantly to marginally reduce by the unprocessed herbal and black teas while a marginal increase was noticed with green tea. Unprocessed rooibos tea significantly ($P < 0.05$) and unprocessed honeybush tea marginally ($P < 0.1$) reduced the total number of foci (>10 µm) of each size category, respectively. The major polyphenolic components, which differ among the teas, may be responsible for the inhibitory effect of the teas on cancer promotion of FB_1 in rat liver.

KEYWORDS: rooibos, honeybush, fumonisin B_1, cancer promotion, rat liver

INTRODUCTION

The popularity of two indigenous herbal teas, rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*), is rapidly increasing as health beverages in South Africa, as well as abroad. Some of the contributing factors to this popularity include the low tannin content, antioxidant properties and natural absence of caffeine (1-6). The main phenolic constituents of rooibos and honeybush teas not only differ from one another but also from those of green and black teas (7-11). The antioxidant (4,5,12-15), antimutagenic and anticlastogenic (16-19) properties of rooibos and honeybush teas are known but little information is available about their cancer modulating properties *in vivo*.

Based on the studies with green and black teas, the use of tea polyphenolic components has been considered as potential chemopreventive nutraceuticals (20,21). Regarding carcinogenesis, tea polyphenols are known to modulate the metabolic fate of carcinogens in several ways rendering them less active/potent thus protecting the target tissue against their adverse effects (22-25). A recent study indicated that the consumption of rooibos and honeybush teas enhanced the activity of phase II

detoxifying enzymes as well as modulated the oxidative status in the liver of rats (26). Cytosolic liver fractions of the herbal tea-treated rats protect against mutagenesis of aflatoxin B_1 (AFB_1) and 2-acetylaminofluorene (2-AAF) while the microsomal mediated activation of AFB_1 was reduced indicating the potential of the tea components to modulate their metabolic fate *ex vivo* (19). These findings suggest that aqueous extracts of rooibos and honeybush teas are likely to modulate the carcinogenic potency of carcinogens *in vivo*.

Several studies in animals showed a reduced cancer incidence after consumption of black and green teas (27,28). The consumption of tea polyphenols and tea pigments comprising of the oxidized products, the theaflavins and thearubigins, significantly reduced the number as well as the average area of $GSTP^+$ foci in the liver (29,30). An aqueous extract of green tea inhibited both cancer initiation and promotion of AFB_1–induced hepatocarcinogenesis in male Fischer rats (31). The fumonisin B (FB_1) mycotoxins were recently characterized as potent liver cancer promoters that occur in maize and showed to synergistically interact with AFB_1 in the induction of liver nodules in a short-term carcinogenesis model (32). As these mycotoxins co-occur naturally the modulation of their carcinogenic properties is of importance (33,34). Numerous studies reported on the modulation of the carcinogenic properties of AFB_1 by natural occurring dietary constituents (23,35,36) while no studies, as yet, have been conducted on the modulation of the carcinogenic properties of the fumonisins.

The present study investigated the chemoprotective properties of aqueous extracts of processed and unprocessed herbal teas as well as green and black teas against the cancer promoting activity of FB_1 in rat liver utilizing diethylnitrosamine (DEN) as initiator with the induction of hepatic preneoplastic foci as endpoint.

MATERIALS AND METHODS

Chemicals Diethylnitrosamine (DEN) was purchased from Sigma Chemical Co (Cape Town, South Africa) and prepared in dimethylsulfoxide (DMSO). The FB_1-containing diet for the rats was prepared by evaporating FB_1, dissolved in methanol (37), on a sub-sample (200 g) of Epol rat mash diet and mixing it with the control diet (Epol rat mash) to obtain the desired dietary level of 250 mg/kg diet. The diet was then stored under nitrogen at 4 °C. The FB_1 was purified according to the method of Cawood *et al.* (38) at the PROMEC Unit (Medical Research Council, Tygerberg, South Africa) to a purity of

93-95%. Glutathione-S-transferase placental form (GSTP) antibody was obtained from DAKO. Reduced (GSH) and oxidized (GSSG) glutathione were purchased from Roche (Cape Town, South Africa). Glutathione reductase, perchloric acid (PCA), tricholoro acetic acid (TCA) 1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate (M2VP), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), NADPH, EDTA, gallic acid, (+)-catechin, quercetin, mangiferin, rutin were purchased from Sigma Chemical Co (Cape Town, South Africa). Phycoerytherin (B-PE) were purchased from ProZyme and 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), Trolox, hesperidin and hesperetin were obtained from Aldrich Chemical Company (Cape Town, South Africa). Aspalathin and nothofagin, with >95% purity were supplied by Ms P Snijman (PROMEC Unit, MRC, South Africa), while iso-vitexin, vitexin, iso-quercitrin and orientin, iso-orientin were purchased from Extrasynthese (Genay, France). All other chemicals used were of analytical grade.

Plant material and preparation. Black tea (*C. sinensis var. assamica*), a blend of locally produced African and Sri Lankan teas was bought from a retail outlet in Cape Town while green tea (*C. sinensis var. sinensis*), imported from China, was a gift from Vital Health Foods (Kuilsriver, South Africa). Processed ("oxidized") and unprocessed (green "unoxidized") rooibos and honeybush (*C. intermedia*) teas were obtained from Dr Joubert from the Agriculture Research Council (ARC), Infruitec-Nietvoorbij, South Africa. All the tea extracts were prepared at concentrations customarily used for tea making purposes (39,40). Freshly boiled tap water was added to the plant material at concentrations of 2 g/100 mL for processed and unprocessed rooibos tea, green and black teas and at 4 g/100mL for processed and unprocessed honeybush tea. The aqueous extracts were allowed to cool to room temperature before these were dispensed into the water bottles. Fresh tea was prepared every second day.

HPLC quantification of the major flavonoids of the rooibos and honeybush tea aqueous extracts. Freeze-dried samples of rooibos and honeybush aqueous extracts were diluted in deionised reverse osmosis water (Modulab Water Purification System from Continental Water System Corporation) to a concentration of ~ 0.38%, filtered (Nylon, 0.45 μm) and analyzed on a LiChrospher 100 RP-18 (5 μm, 250 x 4 mm) column with a C_{18} guard column, using a Merck/Hitachi LaChrom2000 system comprising of a L-7400 detector, L-7100 pump, Rheodyne 7725i injection valve, D-7000 HPLC system manager and interface module. Polyphenols were quantified at 280 nm

according to the method of Joubert (41) with a modification to the initial gradient. Calibration curves were constructed for each flavonoid. Rutin co-eluted with iso-quercitrin and the quantification was expressed as quercetin equivalents.

Treatment of animals. Ninety male Fischer rats, obtained from the Primate Unit, MRC (Tygerberg, South Africa) had free access to rat mash (Epol Ltd, Johannesburg, South Africa). They were housed in wired top and bottom cages fitted with Perspex ^{TM} houses and kept in a controlled environment of 23-24°C, 50% humidity and a 12 hr light cycle. Rats, weighing between 150-170 g, were randomly divided into nine groups of ten rats each and caged individually. Initiation was effected by a single i.p. dose of diethylnitrosamine (DEN; 200 mg/kg body weight). Tea feeding, as the sole source of drinking fluid, commenced one week after initiation until the end of the experiment, while promotion commenced three weeks after initiation by feeding the FB_1-diet (250 mg/kg) for 21 days (Figure 1). The positive control rats received the DEN initiation and FB_1 promotion treatments (DEN-FB_1) in the absence of the tea with tap water being the sole source of drinking fluid. Other control groups received either DEN or DMSO (carrier solvent) treatments with the normal rat diet and tap water as drinking fluid. All the rats were average fed during the FB_1 promotion treatment period according to the daily feed intake of the DEN-FB_1 group receiving tap water. The body weights of the rats were monitored on a weekly basis. The average weekly feed and FB_1 intake was calculated using mean daily rat feed intake as a function of the body weight and expressed as g feed or mg FB_1 per 100 g body weight. At termination, animals were fasted (16 hr), euthanazed by i.p. injection of sodium pentobarbital (0.15 ml /100 g bw) and blood collected from the abdominal aorta. Livers were excised, weighed and sections processed in buffered formalin for histological examination. The remaining liver tissue were immediately frozen in liquid nitrogen and stored at –80°C for biochemical analyses.

Clinical parameters. The clinical biochemical parameters including serum creatinine, total cholesterol, total iron, aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) were measured on a Technicon RA 1000 automated analyzer.

Determination of TBARS. The thiobarbituric acid reacting substances (TBARS) were determined according to a modified method described by Esterbauer *et al.* (42). Sub-samples of the excised livers were homogenized on ice in 19 volumes of 0.01 M

phosphate buffer (pH 7.4) and incubated with 15 μM $FeSO_4$ for one hour at 37°C. The incubated homogenate (1 mL) was mixed with 2 mL of cold trichloro acetic acid (TCA) reagent (10% TCA, 0.01% BHT). The samples were centrifuged (3000 rpm) and 2 mL of the resultant supernatant was combined with 2 mL of 0.67% TBA solution and incubated at 90 °C for 20 min. The mixture was allowed to cool to room temperature and the absorbance was measured at 532 nm. Lipid peroxidation was expressed as nmol malondialdehyde (MDA) per mg protein using a molar extinction coefficient of 1.56 X 10^5 (43). Non-specific lipid peroxidation was prevented by the incorporation of EDTA in the phosphate buffer and BHT in the reaction solution for the TBARS assay.

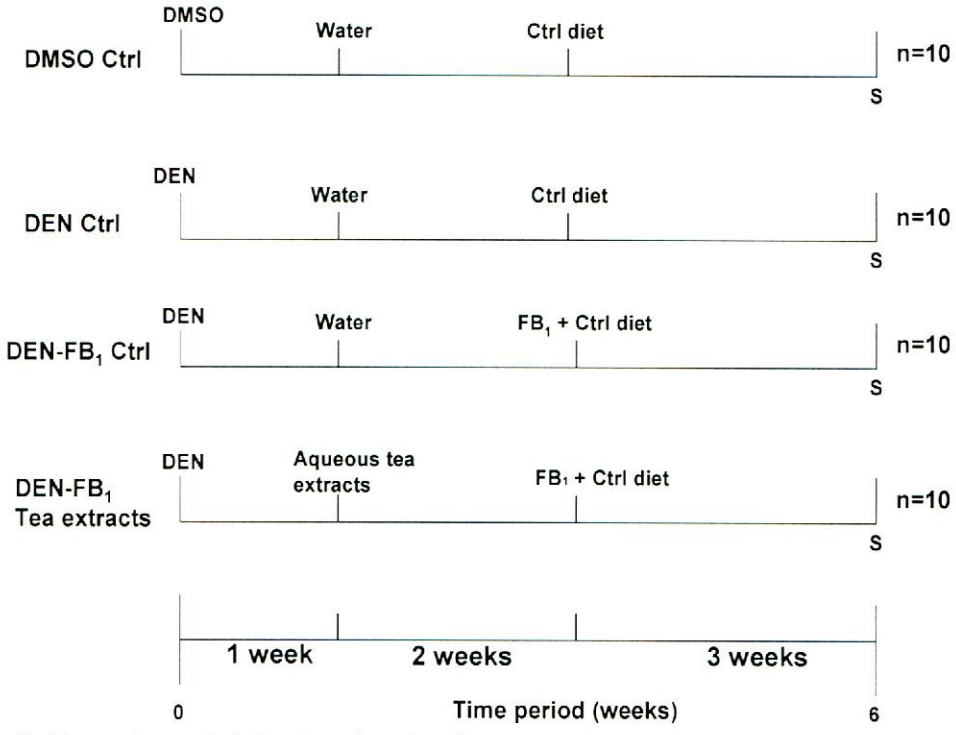


Fig. 1. Experimental design for the liver carcinogenesis model (for details see Materials and Methods). DEN = intra peritoneal initiation regime (200 mg/kg body weight); FB_1 + Ctrl diet = Rat mash containing FB_1 (250 mg/kg diet) as promotion regime; s = sacrificed animals; n = animals per group; ctrl = control.

Oxygen radical absorbance capacity (ORAC). Sub-samples of the stored livers were homogenized in 4 volumes of phosphate buffer (75 mM, pH 7.0) in a Thomas homogenizer (10 strokes) and centrifuged at 12 000 g for 10 min at 4°C. The supernatant was deproteinized using 0.25 M perchloric acid (PCA) and again centrifuged at 16 000 g for 15 min. The resultant supernatants were then stored at -80

°C prior to analysis. The ORAC assay, based on the procedure by Cao and Prior (44) were done in 96 well microtiter plates and consisted of 170 µl of β-PE (80 µg/mL) and 10 µl of diluted sample (1:1) incubated at 37 °C for 15 min. The reaction was initiated by the addition of 20 µl AAPH (240 mM) and the fluorescence (emission 590 nm, excitation 530 nm) recorded every 5 min until the reading had declined to less than 5% of the initial reading. The ORAC values were calculated as described by Cao and Prior (44) and expressed as µM Trolox equivalents/mg wet liver weight.

Glutathione analysis. The total glutathione (GSH and GSSG) was measured according to a modified method of Tietze (45). Liver samples were homogenized (1:10) in 15% (w/v) TCA containing 1 mM EDTA for GSH determination and in 6% (v/v) PCA containing freshly prepared 3 mM M2VP and 1 mM EDTA for GSSG determination on ice. After centrifugation at 100 000 g for 10 min, 50 µl of supernatant was added to glutathione reductase (1 U) and 75 µM DTNB. The reaction was initiated by addition of 0.25 mM NADPH to a final volume of 200 µl. The change in absorbance was monitored at 410 nm for 5 min and levels calculated using pure GSH and GSSG as standards.

GST-Pi immunohistochemical assay. Histochemical staining for the placental form of glutathione-S-transferase (GSTP) was conducted on dewaxed liver sections using a three-stage indirect strepavidin-biotin technique to identify $GSTP^+$ stained hepatocytes (46). The enzyme-altered foci were quantified microscopically (10 X magnification), according to their number and size (internal diameter) and categorized according to the following sizes, 5-10 (mini foci), 11-20, 21-30, >30 µm and expressed as number of foci/cm^2 of the liver section, the area of which was determined by image analyses. The relative amount of each focal size category was expressed as a % of the total $GSTP^+$ foci (>5 µm).

Statistical analysis

Data were tested for normality using the Kolmogorov-Smirnof Test and Levene's Test for Equality of variances. One-way ANOVA's were used to test for significant group differences followed by Tukey-Cramér multiple comparison tests in order to establish which groups differed significantly. The Kruskal-Wallis Test which is a non-paramatric analogue to the one-way ANOVA was used to test for group differences when the data was not normally distributed. Statistical significance was at the 5% level ($p < 0.05$). Statistical comparisons were conducted between 1) the DEN-FB_1 treated rats with the

DEN and DMSO control as well as the tea treated groups and 2) among the tea-treated groups.

RESULTS

Intake of selective herbal tea flavonoids. Aspalathin was the major flavonoid consumed by the rats when fed the unprocessed rooibos tea, followed by its flavone counterparts, orientin and iso-orientin, and the flavonol glycosides, rutin/isoquercitrin. Nothofagin and its flavone counterparts, vitexin and iso-vitexin constitute some of the minor flavonoids consumed (Table I). In the aqueous extract of processed rooibos, aspalathin and nothofagin were reduced and the flavones increased, resulting in an increased intake of iso-orientin and orientin relatively to aspalathin. The xanthone mangiferin and the flavanone hesperidin, were the major flavonoid constituents consumed by the rats treated with the unprocessed and processed honeybush teas, while trace amounts of hesperetin were detected. Mangiferin and hesperidin were markedly less in the processed honeybush tea.

Feed, FB_1, tea intake profiles and body weight gains. During the first week of promotion there were no significant differences in the average weekly feed intake between the different groups (Table II), resulting in a similar intake of FB_1. However, during the second week the DEN-FB_1 treated rats, with and without the different teas significantly ($P < 0.05$) consumed less feed than the DEN and DMSO control groups. The FB_1 intake did not differ among the groups though. During the third week, rats consuming processed and unprocessed rooibos and processed honeybush teas showed a significant ($P < 0.05$) increase in feed intake when compared to the DEN, DMSO and the DEN-FB_1 control groups. The feed and FB_1 intake was also significantly ($P < 0.05$) higher than in the black and green tea treated rats. However, the average FB_1 intake during the FB_1-treatment period did not differ significantly between the groups while the total amount of FB_1 consumed was similar.

When considering the mean weekly body weight gain (BWG) during the FB_1 promotion, rats consuming black tea significantly ($P < 0.05$) increased the BWG when compared to processed rooibos tea treated rats during the first week. During the second week, consumption of the unprocessed honeybush and black teas significantly ($P < 0.05$) reduced the BWG as compared to the processed rooibos tea and the DEN-FB_1 control group. Marked reductions were also noticed with unprocessed rooibos and

green teas. No significant differences were noticed in the BWG between the different groups during the third week.

Table I Flavonoid quantification of aqueous extracts of rooibos and honeybush teas consumed by rats prior to the FB_1 promoting treatment

Rooibos Tea	**Processed**		**Unprocessed**	
Soluble solids (mg/mL)	2.59 ± 0.44		5.36 ± 0.81	
Phenolic compounds	% of soluble solids	Daily intake (mg/100 g BW)**	% of soluble solids	Daily intake (mg/100 g BW)
Aspalathin	1.05 ± 0.04	0.30	16.9 ± 2.76	9.88
Nothofagin	0.09 ± 0.03	0.03	0.72 ± 0.26	0.42
Orientin	0.93 ± 0.02	0.27	1.31 ± 0.36	0.77
Iso-orientin	1.32 ± 0.08	0.38	1.61 ± 0.36	0.94
Vitexin	0.29 ± 0.01	0.08	0.26 ± 0.04	0.15
Iso-vitexin	0.37 ± 0.06	0.11	0.49 ± 0.15	0.29
Rutin/Iso-quercitrin	0.91 ± 0.05	0.26	1.20 ± 0.20	0.70
Honeybush tea	**Processed**		**Unprocessed**	
Soluble solids (mg/mL)	5.96 ± 0.78b		11.78 ± 1.18c	
Phenolic compounds	% of soluble solids	Daily intake (mg/100 g BW)	% of soluble solids	Daily intake (mg/100 g BW)
Mangiferin	1.11 ± 0.15	0.82	6.68 ± 0.84	9.02
Hesperidin	0.37 ± 0.08	0.27	2.63 ± 0.16	3.55
Hesperetin	trace		trace	

All samples used were aqueous extracts prepared as described in the methods. The values in columns represent the mean ± standard deviation of 2-3 repeats of each sample. *Data from Marnewick *et al.*, 2003. BW = body weight. **Mean daily tea intake of the 2-week pre FB_1 period (Table III) was used for determining of daily intake of polyphenolic compounds.

Table II Feed, FB_1, tea intake and body weight gain parameters of the various treatment groups during the three week FB_1 cancer promoting treatment

Period (week)	Feeding parameters and BWG	Control treatments		DEN-FB_1 treatment						
		DMSO	DEN	Water	Rp	Rg	Hp	Hg	Bl	Gr
1	Feed intake (g/100g BW)	7.38 ± 0.70a	7.12 ± 0.21a	5.30 ± 1.44a	5.73 ± 1.32a	5.15 ± 1.83a	5.71 ± 1.43a	5.40 ± 1.61a	5.41 ± 1.37a	6.07 ± 1.56a
	FB_1 intake (g/100g BW)	-	-	0.97 ± 0.02a	1.14 ± 0.29a	0.83 ± 0.19a	1.06 ± 0.14a	0.96 ± 0.23a	1.06 ± 0.23a	1.17 ± 0.35a
	BWG (g)	10.9 ± 6.2a	nd	11.0 ± 7.4a	8.3 ± 4.1a	10.5 ± 4.4a	13.4 ± 5.1a	15.9 ± 8.6a	20.2 ± 6.7b	17.7 ± 7.10a
2	Feed intake (g/100g BW)	6.92 ± 0.29b	7.16 ± 0.17b	6.22 ± 0.39a	6.14 ± 0.83a	6.10 ± 0.89a	6.17 ± 0.82a	5.92 ± 0.84a	5.87 ± 0.79a	5.91 ± 0.82a
	FB_1 intake (g/100g BW)	-	-	1.62 ± 0.07a	1.63 ± 0.06a	1.64 ± 0.05a	1.64 ± 0.06a	1.58 ± 0.06a	1.55 ± 0.06a	1.57 ± 0.06a
	BWG (g)	14.7 ± 6.1a	nd	-5.0 ± 14.4bc	-5.8 ± 5.9bc	-19.4 ± 10.1b	-7.6 ± 5.3b	-21.3 ± 14.6bd	-22.8 ± 5.2bd	-13.7 ± 6.3b
3	Feed intake (g/100g BW)	6.65 ± 0.14a	7.65 ± 0.26a	6.63 ± 0.35a	7.02 ± 0.08b	7.03 ± 0.06b	7.05 ± 0.08b	6.78 ± 0.29ab	6.70 ± 0.03a	6.83 ± 0.04a
	FB_1 intake (mg/100g BW)			1.67 ± 0.10a	1.77 ± 0.02b	1.75 ± 0.02bc	1.76 ± 0.02bc	1.69 ± 0.03ab(c)	1.68 ± 0.01a	1.70 ± 0.01a
	BWG (g)	6.8 ± 4.1a	nd	4.9 ± 5.7a	10.3 ± 3.3a	10.8 ± 7.7a	8.1 ± 3.6a	9.6 ± 6.7a	7.0 ± 5.8a	4.3 ± 3.5a
Ave FB_1 intake	mg/100g BW	-	-	1.51 ± 0.26a	1.57 ± 026a	1.51 ± 0.35a	1.26 ± 0.27a	1.50 ± 0.29a	1.49 ± 0.26a	1.56 ± 0.27a
Total FB_1 intake	mg/100g BW	-	-	30.11	31.31	30.24	31.37	29.99	29.80	31.20
Mean BWG	G	30.0 ± 8.7b	29.6 ± 16.5b	10.9 ± 15.8a	12.8 ± 6.2a	1.2 ± 8.9a	14.0 ± 6.3a	4.3 ± 9.2a	4.3 ± 10.5a	8.3 ± 6.7a

Abbreviations: Rp = processed rooibos, Rg = unprocessed/"green" rooibos, Hp = processed honeybush and Hg = unprocessed/"green" honeybush tea extracts, Bl = black and Gr= green tea, BWG = body weight gain, nd = not determined. Values in the rows represent the mean of ten rats per group ± STD. Values followed by the same letters do not differ significantly. If letters differ then $P<0.05$ while letters in parenthesis then $P<0.1$.

When considering the mean BWG over the FB_1 treatment period, the unprocessed herbal and black teas caused the lowest increase although changes were not significant.

The mean daily water consumption was approximately 10 mL/100 g body weight. Rats consumed significantly ($P < 0.05$) less unprocessed rooibos and black teas during the pre-FB_1 treatment period when compared to the honeybush teas (Table III). During the first week of FB_1 treatment, the rats consumed significantly ($P < 0.05$) less black tea as compared to the other tea treatments. The tea intake was significantly ($P < 0.05$) reduced during the second week in all the tea treated rats. The black tea intake was significantly ($P < 0.05$) and marginally ($P < 0.1$) lower when compared to the unprocessed honeybush and rooibos teas, respectively. No differences in the tea intake profiles were noticed during the third week.

Table III Tea intake profiles of the various treatment groups before FB_1 feeding and during the three week FB_1 cancer promoting treatment

Period (weeks)	Fluid intake (ml/100 g BW) in DEN-FB_1 treated rats						
	Water	Rp	Rg	Hp	Hg	Bl	Gr
Pre-FB_1 intake (2 weeks)	10.1 ± 1.8a	12.3 ± 2.2ab	10.7 ± 2.1a	12.7 ± 2.9b	12.9 ± 2.3ab	10.5 ± 2.6a	12.3 ± 1.9ab
1	nd	11.3 ± 0.9a	11.9 ± 1.8a	14.8 ± 1.5a	13.0 ± 2.0a	10.1 ± 1.9b	12.5 ± 1.7a
2	nd	9.3 ± 1.2a	8.8 ± 1.6(b)	10.2 ± 2.2a	8.6 ± 1.9a	6.2 ± 0.7b	8.9 ± 1.7ab
3	nd	12.4 ± 2.0a	12.0 ± 1.2a	12.1 ± 1.2a	12.8 ± 1.1a	10.9 ± 0.8a	11.5 ± 2.5a

Abbreviations: Rp = processed rooibos, Rg = unprocessed/"green" rooibos, Hp = processed honeybush and Hg = unprocessed/"green" honeybush tea extracts, Bl = black and Gr= green tea, BW = body weight, nd = not determined. Values in the rows represent the mean of ten rats per group ± STD. Values followed by the same letters do not differ significantly. If letters differ then $P<0.05$. Letters in parenthesis then $P<0.1$.

Effect of FB_1 and various tea treatments on total body weight gain and relative liver weight. The total BWG of the rats, during the entire experimental period, was significantly ($P < 0.05$) reduced in the DEN-FB_1 compared to the DMSO and DEN control groups consuming water (Table IV). No significant differences were noticed between DEN-FB_1 control group and those receiving the various tea treatments. However, among the different tea treated groups, the total BWG of the rats consuming the unprocessed rooibos was significantly lower when compared to the processed

rooibos and green tea. The DEN-FB_1 treatment significantly ($P < 0.05$) reduced the relative liver weights when compared to the DMSO and DEN control groups. The DEN-FB_1 treated rats consuming processed rooibos and honeybush teas showed a marginal ($P < 0.1$) decrease and significant increase ($P < 0.05$) in the relative liver weights, respectively. The relative liver weights of rats consuming the processed and unprocessed rooibos was significantly ($P < 0.05$) decreased when compared to the other tea treated rats. A marginal ($P < 0.1$) decrease in the relative liver weight was affected by unprocessed rooibos tea when compared to unprocessed honeybush tea.

Clinical chemistry. Serum levels of AST, ALT, ALP, creatinine and cholesterol were significantly ($P < 0.05$) increased in the DEN-FB_1 control group as compared to the DMSO and DEN control groups (Table IV). Processed and unprocessed rooibos teas markedly to significantly ($P < 0.05$) further increased serum AST levels, respectively. When comparing the different tea treated groups with one another the rooibos teas significantly ($P < 0.05$) increased AST as compared to black and green tea and marginally ($P < 0.1$) to processed honeybush. Unprocessed rooibos and honeybush teas significantly ($P < 0.05$) enhanced the FB_1-induced serum ALT levels while a marked increase was also noticed with processed rooibos. Among the different teas, unprocessed rooibos tea significantly ($P < 0.05$) enhanced ALT when compared to processed honeybush, black and green teas. Rats consuming the different teas did not alter the FB_1-induced increased serum ALP levels although processed honeybush teas markedly elevated the level. Within the different tea treated rats, processed honeybush marginally ($P < 0.1$) increased ALP when compared to processed rooibos and significantly ($P < 0.05$) to black tea. Unprocessed rooibos tea also marginally ($P < 0.1$) increased ALP as compared to black tea.

Black tea and processed honeybush teas significantly ($P < 0.05$) decreased the serum creatinine levels when compared to the DEN-FB_1 control rats while a marginal ($P < 0.1$) reduction was obtained with unprocessed honeybush tea. A similar trend was noticed when the creatinine level was compared among the different tea consuming groups. With respect to the FB_1-induced increase of serum cholesterol, processed and unprocessed honeybush and green teas significantly ($P < 0.05$) and black tea marginally ($P < 0.1$) decreased the level. Among the different teas, processed and unprocessed honeybush and green teas significantly ($P < 0.05$) lower the cholesterol levels.

Table IV Effect of DEN-FB_1 and various tea treatments on the total body weight gain, relative liver weight and clinical chemistry

Groups	Body weight gain (g)*	Relative liver weight (g)#	AST (U/L)	ALT (U/L)	ALP (U/L)	Creatinine (umol/L)	Cholesterol (mmol/L)	Total Iron (μmol/L)
DMSO	86.9 ± 8.2a	2.9 ± 0.35a	88.1 ± 16.8a	53.7 ± 7.3a	168.9 ± 32.8a	69.0 ± 6.0a	1.6 ± 0.2a	23.1 ± 5.8a
DEN	77.7 ± 25.1a	3.51 ± 0.27c	102.8 ± 15.4a	96.7 ± 11.5c	272.4 ± 69.1c	67.3 ± 3.7a	1.4 ± 0.3a	25.9 ± 2.6a
DEN-FB_1	39.8 ± 13.8b	2.50 ± 0.16b	258.7 ± 60.2b	211.6 ± 48.0b	282.4 ± 82.0b	85.8 ± 4.7b	3.5 ± 0.4b	24.4 ± 7.4a
DEN-FB_1-Rp	44.0 ± 6.8bc	2.36 ± 0.12(b)d	320.6 ± 78.1bd	296.6 ± 113.4be	283.4 ± 61.3bd	85.3 ± 4.4bd	3.3 ± 0.4bc	21.9 ± 2.3ab
DEN-FB_1-Rg	29.6 ± 9.6bd	2.44 ± 0.09bdf	341.1 ± 50.0cd	321.3 ± 75.4def	325.3 ± 86.8bde	83.5 ± 5.8bd	2.9 ± 1.0bce	22.6 ± 4.4ab
DEN-FB_1-Hp	37.9 ± 12.7b	2.79 ± 0.05ae	242.9 ± 54.3b(d)	236.3 ± 63.9beg	355.3 ± 77.8b(d)ef	77.2 ± 3.8cef	2.3 ± 0.8ade	27.5 ± 2.2acd
DEN-FB_1-Hg	36.1 ± 16.4b	2.58 ± 0.19be(f)	283.2 ± 52.1b	280.9 ± 71.7de	314.3 ± 57.9bde	80.8 ± 5.8(b)df	2.1 ± 0.3ad	28.3 ± 4.9acd
DEN-FB_1-Bl	40.2 ± 19.8b	2.58 ± 0.13be	207.1 ± 56.3be	216.0 ± 90.5beg	245.0 ± 58.4bd(e)g	78.4 ± 2.2c(d)f	3.0 ± 0.6(b)cef	24.1 ± 6.3abd
DEN-FB_1-Gr	48.3 ± 13.3bc	2.59 ± 0.11be	226.4 ± 22.5be	229.3 ± 41.4beg	296.8 ± 62.1bde	83.6 ± 3.0bd	2.4 ± 0.5ade(f)	27.1 ± 4.4a(b)d

Values in columns represent the mean ± STD dev. #relative liver weights equal liver weight/body weight. Abbreviations: Rp = processed rooibos, Rg = unprocessed/"green rooibos, Hp = processed honeybush and Hg = unprocessed/"green" honeybush tea extracts, Bl = black and Gr = green tea. Values followed by the same letters do not diffe significantly. When letters differ then $P<0.05$. Letters in parenthesis then $P<0.1$. *Including DEN, Pre-FB_1 tea treatment and FB_1 treatment regimens. n= 7-10 rats per group.

The total iron levels were not significantly altered by the teas as compared to the DEN-FB_1 control group. However, both unprocessed and processed honeybush teas significantly ($P < 0.05$) and green tea marginally ($P < 0.1$) increased the total iron levels as compare to the unprocessed and processed rooibos teas.

Table V Effect of unprocessed and processed herbal, green and black teas on reduced glutathione (GSH), oxidized glutathione (GSSG), the ratio GSH:GSSG, oxidative capacity (ORAC) and lipid peroxidation in livers of rats

Groups	ORAC (uM Trolox eq/mg protein)	GSH (uM/mg protein)	GSSG (uM/mg protein)	GSH:GSSG Ratio	TBARS (nmol MDA /mg protein)
DMSO	15.5 ± 2.4a	3.2 ± 0.69a	0. 21 ± 0.03a	15.1 ± 2.3a	0.08 ± 0.01a
DEN	12.8 ± 2.2b	3.0 ± 0.76a	0.26 ± 0.06(a)	12.1± 3.8a	0.07 ± 0.01a
DEN-FB_1	13.6 ± 0.7b	4.6 ± 0.87b	0.22 ± 0.03a	21.6 ± 4.7b	0.32 ± 0.08b
DENFB_1-Rp	14.5 ± 1.5b	3.2 ± 0.91ac	0.20 ± 0.06ac	17.0 ± 4.4(b)c	0.21 ± 0.07a
DEN-FB_1-Rg	14.7 ± 1.3(b)	2.9 ± 0.91ad	0.26 ± 0.04bde	11.1 ± 3.5ad	0.30 ± 0.04b
DEN-FB_1-Hp	14.4 ± 1.1b	4.7 ± 0.28b	0.22 ± 0.06ace	22.9 ± 7.4bc	0.16 ± 0.04ac
DEN-FB_1-Hg	13.2 ± 1.1b	4.7 ± 1.1b	0.26 ± 0.06(a)(c)eg	19.3 ± 7.6bc	0.26 ± 0.06ad
DEN-FB_1-Bl	13.6 ± 1.1b	5.1 ± 2.2b(c)(d)	0.32 ± 0.05bd(c)g	15.8 ± 6.1(b)c(d)	0.35 ± 0.07be
DEN-FB_1-Gr	10.0 ± 0.88c	4.5 ± 1.2b(c)	0.36 ± 0.09bdf(g)	13.4 ± 5.2ac	0.31 ± 0.09bd

Values in columns represent average of 5 to 8 values per group ± STD. Means followed by the same letter do not differ significantly, when letters differ then $P<0.05$. Letters in parenthesis then $P<0.1$. Abbreviations: Rp = processed rooibos, Rg = unprocessed/"green" rooibos, Hp = processed honeybush and Hg = unprocessed/"green" honeybush tea extracts, Bl = black and Gr = green tea.

Effect on hepatic oxidative status

(i) Oxygen Radical Absorbance Capacity (ORAC). Both the DEN-FB_1 and DEN treatments significantly lowered the hepatic ORAC status when compared to the DMSO control group (Table V). Green tea further decreased ($P < 0.05$) the hepatic ORAC values while unprocessed rooibos tea marginally ($P < 0.1$) increased the ORAC. No significant changes were noticed in the ORAC levels with the other teas although processed rooibos and honeybush teas markedly (not significantly) increased the level when compared to the DEN-FB_1 control rats.

(ii) Hepatic glutathione. Rats receiving the DEN-FB_1 dietary treatment significantly ($P < 0.05$) elevated the GSH levels in the liver when compared to the DMSO and DEN control groups (Table V). Consumption of processed and unprocessed rooibos teas resulted in a significant ($P < 0.05$) reduction in hepatic GSH levels. A similar effect was noticed when the GSH level was compared among the different tea-treated rats. When considering the GSSG levels, rats treated with DEN-FB_1 exhibited no significant changes when compared to the DMSO control group, while a marginal ($P < 0.1$) increase was noticed in the DEN control group. However, hepatic GSSG levels were significantly ($P < 0.05$) elevated with green, black and unprocessed rooibos teas and marginally ($P < 0.1$) with the unprocessed honeybush tea. Green and black teas significantly ($P < 0.05$) and marginally ($P < 0.1$) increased GSSG, respectively, among the different teas. Unprocessed rooibos and honeybush teas significantly ($P < 0.05$) to marginally ($P < 0.1$) increased GSSG, respectively, as compared to their processed counterparts. DEN-FB_1 treatment significantly ($P < 0.05$) increased the GSH:GSSG ratio in the liver when compared to the DMSO and DEN control groups. Rats consuming unprocessed rooibos and green teas significantly ($P < 0.05$) decreased the GSH:GSSG ratio, while a marginal ($P < 0.1$) decrease was noticed with processed rooibos and black teas. Among the different teas, unprocessed rooibos significantly ($P < 0.05$) decreased the ratio.

Lipid peroxidation. The TBARS levels were significantly ($P < 0.05$) increased in the liver of the DEN-FB_1 treated rats (Table V) when compared to the DMSO and DEN groups. The DEN-FB_1 treated rats consuming processed rooibos, processed and unprocessed honeybush teas showed a significant ($P < 0.05$) decrease in the hepatic TBARS levels when compared to the DEN-FB_1 control rats. Consumption of unprocessed rooibos, black and green teas showed no effect on the FB_1-induced lipid peroxidation, although black tea markedly enhanced the level. Among the different teas, processed rooibos and honeybush teas significantly decreased lipid peroxidation when compared to their unprocessed counterparts. Unprocessed honeybush significantly ($P < 0.05$) reduced lipid peroxidation when compared to black tea.

Table VI Effect of various tea treatments on the induction of GSTP$^+$ foci by combined treatment of DEN and FB_1.

Treatment groups	GSTP$^+$ Liver Foci								
	No foci/cm^2 5-10 μm	% of Total	No foci/cm^2 11-20 μm	% of Total	No foci/cm^2 21-30 μm	% of Total	No foci/cm^2 >30 μm/cm^2	% of Total	Total no of foci (>5 μm)
DMSO	Nd	nd	nd	Nd	nd	nd	nd	nd	nd
DEN	4.7 ± 1.7a	58.7 ± 13.4a	1.9 ± 0.8a	32.9 ± 7.6a	0.5 ± 0.8a	6.5 ± 8.7a	0.14 ± 0.34a	2.0 ± 4.0a	6.0 ± 2.4a
DEN-FB_1	8.0 ± 2.8b	23.7 ± 7.5b	18.2 ± 5.6b	50.2 ± 8.4b	6.5 ± 1.4b	19.6 ± 5.0b	2.3 ± 1.4b	6.5 ± 3.9b	35.0 ± 11.1b
DEN-FB_1-Rp	15.9 ± 8.7(b)d	39.2 ± 14.3cd	15.5 ± 5.7bd	39.1 ± 4.3ac	6.5 ± 2.9b	16.1 ± 3.6bc	2.3 ± 1.1bc	6.0 ± 3.2bc	40.1 ± 14.0bd
DEN-FB_1-Rg	12.6 ± 4.9cdf	53.9 ± 17.1a(d)	10.4 ± 5.3c(d)e	36.0 ± 9.0ace	3.1 ± 2.4c	10.3 ± 6.6a(c)d	0.7 ± 1.1ad	1.9 ± 2.8ad	25.4 ± 10.3(b)el
DEN-FB_1-Hp	17.9 ± 5.4cd(f)	42.3 ± 7.9ad	15.1 ± 6.3bde	33.9 ± 4.6ade	7.6 ± 4.8bd	16.4 ± 7.0bc(d)e	3.5 ± 2.7bc	7.5 ± 4.1b	44.2 ± 16.3bg
DEN-FB_1-Hg	19.7 ± 8.5cd(f)	52.7 ± 14.7a(d)	11.9 ± 5.9(b)deg	31.8 ± 6.2ade	4.0 ± 2.7c(d)	10.6 ± 5.1a(c)d	0.9 ± 1.7(b)(c)d	2.3 ± 4.1(b)d	38.0 ± 14.2b(f)
DEN-FB_1- Bl	30.0 ± 3.5ce	54.0 ± 12.3a(d)	17.3 ± 9.9bd(e)	27.9 ± 15.8ace	7.7 ± 3.0b	13.8 ± 6.1(b)cde	2.5 ± 1.4bc(d)	4.3 ± 2.1bde	57.5 ± 11.5cg
DEN-FB_1.Gr	23.9 ± 11.3cdg	37.8 ± 13.3ad	18.2 ± 6.2bdf(g)	33.3 ± 4.6ade	10.4 ± 6.6b	17.5 ± 8.8bc(d)e	5.9 ± 5.2b(c)(d)	9.7 ± 7.2(b)ce	61.8 ± 14.4c(g)

Abbreviations: Rp = processed rooibos, Rg = unprocessed/"green" rooibos, Hp = processed honeybush and Hg = unprocessed/"green" honeybush tea extracts, Bl = black and Gr = green tea, nd = not detected. Values in columns represent the average of ten rats per group ± STD. Values followed by the same letter then no significance differences are shown. When letters differ then $P<0.05$, while letters in parenthesis then $P<0.1$.

GSTP liver foci

Differences between DEN-FB_1 and various tea treatments. FB_1 significantly ($P < 0.05$) increased the number of $GSTP^+$ foci in all size categories as reflected in the increased total number when compared to the DEN control rats. No $GSTP^+$ foci were detected in the liver of the DMSO control rats. (Table VI). All the tea preparations significantly ($P < 0.05$) to marginally ($P < 0.1$) increased the number of mini foci (5-10 μm) when compared to the DEN-FB_1 treatment. A similar effect was noticed when considering the relative number of foci, expressed as a % of the total. Of interest is that the DEN control group, unprocessed rooibos and honeybush and black teas numerically exhibited the highest % of mini foci. When considering the focal size 11 to 20 μm, which constitutes 50% of the total number of foci in the DEN-FB_1 treated group, the unprocessed rooibos and honeybush teas significantly ($P < 0.05$) and marginally ($P < 0.1$) decreased the number of foci, respectively. The relative amount was significantly ($P < 0.05$) decreased by all the tea-treated groups. In the focal size category 21-30 μm, which constitutes approximately 20% of the total number of foci, the unprocessed herbal teas again significantly ($P < 0.05$) decreased the number of foci while the relative amount was significantly ($P < 0.05$) and marginally ($P < 0.1$) decreased by the unprocessed herbal teas and black tea, respectively. When considering the focal size greater than 30 μm, constituting approximately 6% of the total amount of foci, the unprocessed rooibos tea significantly ($P < 0.05$) and unprocessed honeybush tea marginally ($P < 0.1$) decreased the number. A similar response was obtained when considering the relative amount, although green tea showed a marginal ($P < 0.1$) increase. Of the herbal tea treated groups unprocessed rooibos tea marginally ($P < 0.1$) reduced the total number of $GSTP^+$ foci (>5 μm), while the green and black teas showed a significant ($P < 0.05$) increase when compared to the DEN-FB_1 control group. However, when omitting the mini-foci (5 to 10 μm), none of green and black teas exhibited a significant increase while unprocessed rooibos and honeybush significantly ($P < 0.05$) and marginally ($P < 0.1$) reduced the total number of foci, respectively (Figure 2).

Differences between tea treatment groups. Among the different tea treated groups, black tea exhibited the highest number of mini foci while a marginal ($P < 0.1$) increase was noticed for the honeybush teas. The relative amount of mini foci was marginally ($P < 0.1$) increased by the unprocessed herbal teas as compared to the processed teas. In contrast black tea marginally ($P < 0.1$) increased the relative amount of mini foci when

compared to green tea. In the focal size category 11-20 µm, the unprocessed rooibos marginally ($P < 0.1$) decreased the number of foci as compared to the processed rooibos and black teas and significantly ($P < 0.05$) when compared to green tea. Unprocessed and processed honeybush also marginally ($P < 0.1$) to significantly ($P < 0.05$) decreased the number of foci as compared to black and green teas, respectively. When considering the relative amount, the rooibos teas accounted for the highest contribution to this size category. Unprocessed rooibos and honeybush significantly ($P < 0.05$) reduced the number of foci in the size category of 21-30 µm when compared to their processed counteracts as well as the black and green teas. With respect to the relative amount, the unprocessed herbal teas marginally ($P < 0.1$) lowered the level when compared to their processed counterparts and green tea. A similar effect was noted in the focal size >30 µm, where the unprocessed rooibos tea significantly ($P < 0.05$) reduced the number as compared to the processed herbal teas. A marginal ($P < 0.1$) reduction was also noticed when compared to the green and black teas. Unprocessed honeybush tea also marginally ($P < 0.1$) reduced the number of foci as compared to the processed counterpart and green and black teas. Unprocessed rooibos significantly ($P < 0.05$) reduced the relative amount, when compared to the processed herbal tea and green teas. Unprocessed honeybush tea showed a significant ($P < 0.05$) and marginal ($P < 0.1$) reduction when compared to green tea and processed honeybush, respectively.

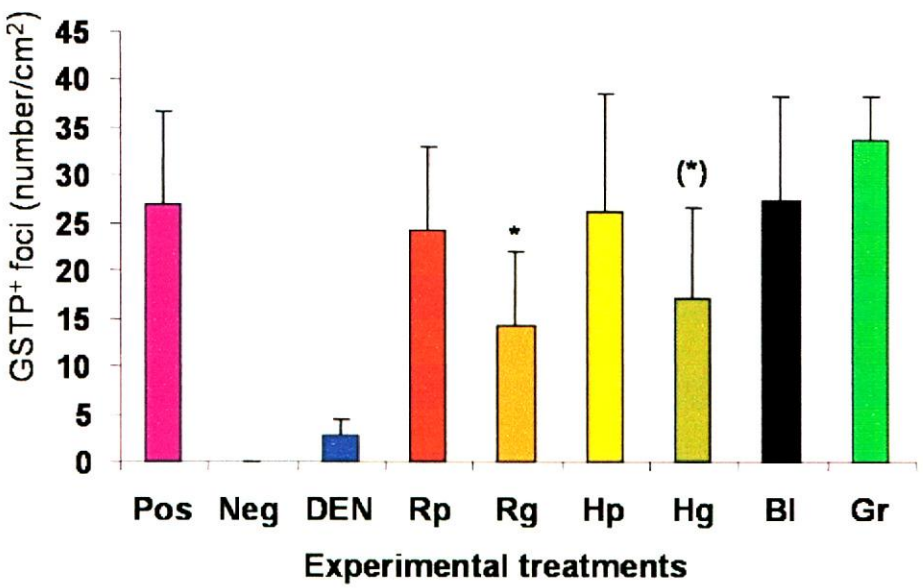
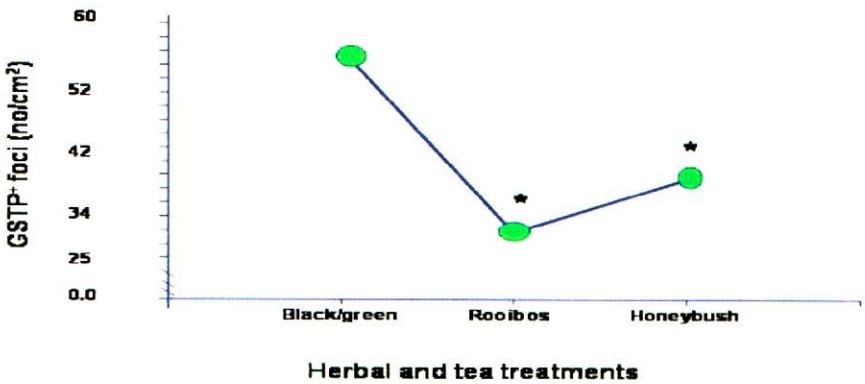


Fig. 2. The total number of $GSTP^+$ foci (>10 µm) in liver sections of rats consuming the different teas while fed a dietary level of 250 mg FB_1/kg diet for 3 weeks. Values represent the means ± STD of 10 rats per group.

The herbal teas significantly ($P < 0.05$) decreased the total amount of foci (>5µm) as compared to the black and green teas when the combined effects were considered (Figure 3A). The separate effects of the different teas indicated that unprocessed rooibos and honeybush significantly ($P < 0.05$) decreased the total number of foci when compared to the green and black teas (Figure 3B). A similar effect was noticed with processed herbal teas.

A



B

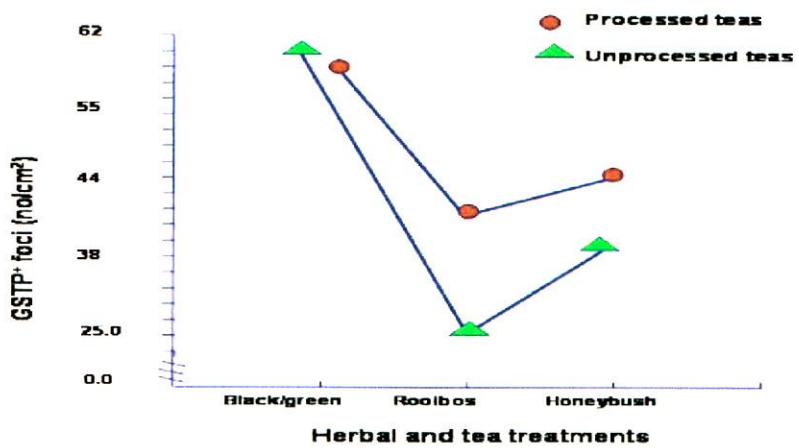


Fig. 3. (A) Interactive plots illustrating the combined effects of the various tea treatments on the induction of total number of hepatic $GSTP^+$ foci (>5 µm) by FB_1 (*significant difference from black and green tea), (B) Separate effects of the tea processing on the induction of $GSTP^+$ foci (For statistical differences see Table VI).

DISCUSSION

FB_1 has been characterized as a non-genotoxic liver cancer promoter (47) and subsequently shown to be hepatocarcinogenic in mice (48) and rats (49) and nephrocarcinogenic in rats (48). The mechanism of cancer induction is not known at present but studies in rats indicate that FB_1 could effect both cancer initiation and promotion in the liver (37,50,51). The disruption of growth-stimulatory responses in normal and altered initiated cells is suggested to be important in establishing a growth differential whereby the preneoplastic cell populations are clonally expanded, subsequently leading to hepatocarcinogenesis induced by FB_1 (52,53). At a cellular level, FB_1 also disrupts sphingolipid, phospholipid and fatty acid metabolism which have been suggested to be the underlying mediators responsible for the growth differential created in the liver (54-56).

The cancer promoting potential of different dietary levels of FB_1 in DEN-initiated rats has been previously established (51). The present study confirms that, at the dietary level used, FB_1 induced hepatotoxicity as indicated by a significant ($P < 0.05$) increase in the liver function enzymes and cholesterol, with a resultant decrease in body weight gain and the relative liver weight (51,57). Serum creatinine, a marker for kidney damage effected by FB_1 (56), was also increased. Black and processed honeybush teas significantly ($P < 0.05$) and unprocessed honeybush tea marginally ($P < 0.1$) reduced the serum creatinine, suggesting a protective effect against FB_1-induced nephrotoxicity. The honeybush teas and green tea also effectively protected against FB_1-induced accumulation of serum cholesterol. The high level of hesperidin in honeybush tea is of interest in this regard as hesperetin, the aglycone of hesperidin, is known to reduce the level of plasma cholesterol in high cholesterol fed rats (58). Consumption of unprocessed rooibos and to a certain extent, processed rooibos and unprocessed honeybush teas, further increased the clinical chemical parameters for liver damage induced by FB_1, suggesting an enhanced hepatotoxic effect. This resulted in a reduction in the relative liver weight ($P < 0.05$) of the rats consuming the processed and unprocessed rooibos teas when compared to the other teas. As the tea intake profiles during the pre-FB_1 and FB_1 treatment periods were similar, except for the black teas which tended to be lower, and total FB_1 intake during this period did not differ, specific interactions between the tea constituents and FB_1 are likely to affect the enhanced hepatotoxicity. The latter is further substantiated that consumption of the teas

did not induce any hepatotoxic effects in the liver in the absence of FB_1 under similar experimental conditions (26).

One possibility for the enhanced hepatotoxicity of FB_1 could be the mobilization of iron in the liver, shown to be effected by FB_1 (53), resulting in a possible prooxidant effect when interacting with the herbal tea polyphenolic compounds. In the present study the total plasma iron was significantly ($P < 0.05$) increased by the honeybush teas and marginally ($P < 0.1$) by green tea when compared to the rooibos teas. This observation was mainly due to a marked reduction in the total plasma iron in the rooibos treated rats as compared to the DEN-FB_1 group consuming water. This would imply that the herbal teas, as well as the green tea, interfere with the iron mobilization effect enhanced by FB_1 in the liver and therefore could modulate the oxidative status in the liver. With respect to oxidative damage, processed rooibos and unprocessed and processed honeybush teas significantly ($P < 0.05$) reduced the FB_1-induced lipid peroxidation under the present experimental conditions. This finding is in agreement with the antioxidant potency of these teas which is known to be less than that of unprocessed rooibos, black and green teas (4,14) and therefore less likely to interact with iron to induced a prooxidant effect. The current study supports this finding as the processed herbal teas exhibited a significant ($P < 0.05$) lower inhibitory effect on lipid peroxidation. Of interest was the significant ($P < 0.05$) increase in lipid peroxidation by green and black tea as compared to the honeybush teas and unprocessed rooibos teas.

Another contributing factor is the ORAC, which was only marginally ($P < 0.1$) increased by unprocessed rooibos and markedly by processed rooibos and honeybush teas, suggesting an enhanced protective effect against oxidative damage. This apparent contradictory finding with respect to the increased serum liver function enzymes and enhanced protection against lipid peroxidation could be related to the stabilizing effect of the herbal teas on the ORAC values as well as the hypothesis that lipid peroxidation is a secondary event following FB_1-induced cytotoxicity (59). Therefore, despite the apparent increased cytotoxicity of FB_1 the subsequent lipid peroxidation is prevented by rooibos and honeybush teas. Although the herbal teas did not enhance the ORAC levels in rats (26) it stabilized the levels during FB_1 promotion in the present study, which could explain the enhanced protective effects on lipid peroxidation. As rats consuming the unprocessed rooibos tea are exposed to far higher levels of flavonoids, possible interactions with iron prohibited any significant reduction in lipid peroxidation as

compared to the processed tea. The lower antioxidant potency of unprocessed honeybush tea (13,14), therefore, could explain the higher inhibitory effect on the FB_1-induced lipid peroxidation. No effect was obtained with black tea on the ORAC level and FB_1-induced lipid peroxidation while green tea, significantly ($P < 0.05$) further reduced the ORAC level while lipid peroxidation was not effected. A possible interaction with the green tea polyphenolic compounds and FB_1-mobilized hepatic iron could be responsible for the reduced ORAC level. A reduction of ORAC by green tea was also reported previously when rats were consuming the tea over a period of 10 weeks (26). This would suggest that, when considering ORAC, the FB_1 treated rats consuming green tea were subjected to additional stress.

Apart from the ORAC, the GSH:GSSG ratio is also an important oxidative parameter during promotion as the liver is subjected to a variety of stresses, some of which may lead to a depletion of GSH levels. FB_1 is known to increase oxidative stress in the liver (59) and a recent study in glioblastoma cells showed that FB_1 reduced the level of GSH (60). However, the decrease in GSH during stressed conditions is likely to be counteracted by the development of gamma glutamyl-transpeptidase positive (GGT^+) foci. These altered hepatocytes are provided with amino acids necessary for GSH synthesis by GGT, located on the outer surface, which primarily acts as a glutathionase (61). The GGT^+ cells will therefore be able to replenish glutathione providing the cell with a selective growth advantage during promotion. A study, utilizing a DEN carcinogenesis model, also reported on an increased GSH level in the liver of male Wistar rats, which was ascribed to the induction of GGT^+ adenomas (62). In the present study, rats treated with DEN-FB_1 significantly increased the level of GSH in the liver, presumably due to the increased number of preneoplastic foci, known to stain positive for GGT (47,49), resulting in an increased GSH:GSSG ratio. However, the single DEN treatment regimen did not increase the GSH level as only few enzyme altered foci were induced in the absence of the FB_1 promoting treatment. The increased level of GSH was significantly ($P < 0.05$) lowered by processed and unprocessed rooibos teas, resulting in a decreased GSH:GSSG ratio. Black and green teas marginally ($P < 0.1$) to significantly ($P < 0.05$) decreased the ratio, respectively, mainly due to an increased GSSG level. It is known that, under stressed conditions, this ratio tends to decrease either due to increased GSSG or decreased GSH levels (63). The green tea probably enhanced the oxidative stress in the liver of rats exposed to FB_1 as the GSSG level was significantly increased without affecting the GSH level, thereby decreasing the

GSH:GSSG ratio. This hypothesis is further strengthened as green tea also reduced ORAC in the liver of these rats. The unprocessed rooibos tea, however, decreased the ratio as a result of the decrease in GSH, which could be explained, as discussed above, by the reduced number of altered foci in the liver. An interesting relationship exists between the GSSG status and the inhibition of lipid peroxidation. Teas exhibiting the highest protective effect against lipid peroxidation were also associated with the lower level of GSSG in the liver, suggesting that the accumulation thereof, as stated above, is related to an increased oxidative stress.

Consumption of the different teas as a sole source of drinking fluid significantly ($P < 0.05$) arrested the proliferation of $GSTP^+$ altered cells in the presence of the cancer promoter FB_1. This was deduced from the finding that the relative number of foci (10-20 μm) that constitutes approximately 50% of the total amount of foci, significantly ($P < 0.05$) decreased in liver of rats treated with the different teas. In contrast, the relative number of mini foci (5-10 μm) was significantly ($P < 0.05$) increased, suggesting that the different teas interfere with the promoting of these lesions by FB_1. Unprocessed rooibos and honeybush teas also significantly reduced the relative number of the larger foci, further suggesting an interference with cancer promotion. The green tea flavonol, (-)-epigallocatechin-3-gallate (EGCG) was shown to inhibit the proliferation of a hepatoma cell line through the induction of apoptosis and cell cycle arrest (64). Inhibition of cell proliferation was suggested in a study where green tea constituents and polyphenols inhibited the number and size of enzyme altered foci when administered to rats during cancer promotion (31). In the present study all the teas delayed the development of DEN-induced preneoplastic cells presumably due to the inhibition of cell proliferation. However, the reduction of the number of foci, as was noticed with the unprocessed rooibos tea and to a certain extent with the unprocessed honeybush tea, could be related to the increased toxicity which could further enhance the apoptotic effects of FB_1 in the liver and thereby inhibited the growth of preneoplastic lesions. It was previously reported that apoptosis, induced by FB_1, delayed cancer induction by removing initiated cells from the liver (49,50).

The antioxidant activity of many plant phenolic components may play an important role in maintaining the balance between cell growth and cell death in preneoplastic lesions (65). In the present study, no conclusive evidence exists to show the involvement of antioxidant activities of the different teas in the decreased number

and/or delayed development of $GSTP^+$ foci. However, processing of the herbal teas, known to reduce the antioxidant status (14,66), also reduced the inhibitory effect on FB_1-induced cancer promotion. Varying effects on ORAC, GSH levels, and the inhibition of lipid peroxidation were noticed which could be related to the differences in polyphenolic constituents among the various teas and their specific interactions with FB_1 during cancer promotion. Therefore, the protection against FB_1-induced cancer promotion by the different teas may not be entirely related to their antioxidative activities. Other features, e.g. their pro-oxidant activity and effect on cell regulatory processes such apoptosis may also be involved and need to be investigated. Tea polyphenols have been shown to exhibit pro-oxidant features and have been associated with the induction of apoptosis in cultured cancer cells (67). The mechanisms involved in the inhibitory effect against liver tumour promotion by the herbal teas are therefore of interest and remain to be elucidated.

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CHAPTER 7

Inhibition of Tumour Promotion in Mouse Skin by Extracts of Rooibos (*Aspalathus linearis*) and Honeybush (*Cyclopia intermedia*), Unique South African Herbal Teas

Cancer Letters (in press)

Inhibition of tumour promotion in mouse skin by extracts of rooibos (A*spalathus linearis*) and honeybush (C*yclopia intermedia*), unique South African herbal teas

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ABSTRACT

Ethanol/acetone (E/A) soluble fractions, prepared from methanolic extracts of processed and unprocessed rooibos and honeybush as well as green tea, significantly ($P < 0.001$) reduced tumour formation in mice skin initiated with 7,12-dimethyl-benz[a]anthracene (DMBA) followed by 12-O-tetra-decanoylphorbol-13-acetate (TPA) promotion. The green tea E/A fraction completely inhibited (100%) tumour formation followed by fractions of unprocessed honeybush (90%), processed honeybush (84.2%), processed rooibos (75%) and unprocessed rooibos (60%). No relationship exists between the total polyphenol (TP) content of the herbal tea fractions, inhibition of microsomal lipid peroxidation and the inhibition of skin tumour promotion. The green tea fraction, with the highest flavanol/proanthocyanidin content, also exhibited the highest protective activity against hepatic microsomal lipid peroxidation, and completely inhibited skin tumour formation. The unprocessed rooibos tea fraction, with a similar TP content exhibited the highest inhibition against lipid peroxidation of the herbal tea fractions, but showed the weakest protection against tumour promotion. In contrast, the unprocessed honeybush tea fraction with a similar TP content and inhibitory effect on lipid peroxidation than the processed rooibos fraction, exhibited the highest protection against tumour promotion. The flavanol/proanthocyanidin and flavonol/flavone contents of the unprocessed honeybush tea fraction, expressed as a percentage of the TP, were similar to that of the green tea and unprocessed rooibos fractions, respectively. Differences in the flavanol/proanthocyanidin and flavonol/flavone composition and/or non-polyphenolic constituents are likely to be important determinants in the inhibition of tumour promotion by the herbal tea E/A fractions in mouse skin.

KEYWORDS: Rooibos, Honeybush, TPA, Chemoprevention, Tumour promotion

INTRODUCTION

Chemical carcinogenesis in murine and possibly human skin is a multi-step process including initiation, promotion and progression [1,2]. In mouse skin, a single topical application of 7,12-dimethylbenz[*a*]anthracene (DMBA) affects initiation while promotion is accomplished by repeated topical applications of a promoter such as 12-O-tetradecanoylphorbol-13-acetate (TPA) [3,4]. In contrast to initiation, which is irreversible and possibly unavoidable because of continuous exposure to chemical and physical carcinogenic agents, the process of promotion is reversible [4,5]. The reversibility of tumour promotion therefore provides an opportunity to interrupt or delay the development of altered lesions resulting in tumour formation.

A variety of plant and/or other phenolic compounds exhibit chemoprotective properties by disrupting the different stages of multi step skin carcinogenesis, especially tumour promotion [5-11]. The phenolic composition of green tea has been well characterised and the major flavonoid constituents, the catechins, are known to be inhibitors of cancer initiation and promotion in mouse skin [11-13]. The major phenolic components of the unprocessed South African herbal teas are the dihydrochalcones, aspalathin and nothofagin present in rooibos (*Aspalathus linearis*) and the xanthone, mangiferin and flavanone, hesperidin in honeybush (*Cyclopia intermedia*) [14,15]. In the processed herbal teas, aspalathin remains one of the major components in rooibos. Although mangiferin and hesperedin decrease with processing, they also remain the major monomeric polyphenols in processed honeybush. The polyphenolic composition differs from that of green tea and their cancer protective properties are not known at present.

Aqueous extracts of rooibos tea significantly decreased the number of chromosomal aberrations in hamster ovary cells treated with benzo[a]pyrene in the presence of liver homogenates [16]. An *in vivo* study in male ICR mice showed a significant decrease in the number of micronucleated reticulocytes, induced by mitomycin C, by aqueous extracts of rooibos tea [16]. Apart from these studies very little is known about the modulation of the genotoxic effects of carcinogens by these herbal teas. Recent investigations showed that aqueous extracts of honeybush and rooibos exhibit antimutagenic properties against aflatoxin B_1- and 2-acetylaminofluorene-induced mutagenesis *in vitro* [17] and *ex vivo* [18]. Aqueous extracts of unprocessed and processed rooibos as well as unprocessed honeybush significantly enhanced the

antioxidant capacity in the liver of rats by stabilising glutathione (GSH) [19]. The activity of hepatic phase II metabolising enzymes, glutathione-S transferase (GST-α) and UDP-glucuronosyl transferase (UDP-GT) was also significantly enhanced in the liver by the aqueous extracts of rooibos and honeybush teas. This would imply that these herbal teas not only altered the metabolic fate of carcinogens but also the oxidative status of cells that can protect against the adverse effects of oxidative damage induced by many carcinogens. The present study investigated the modulating effect of ethanol/acetone (E/A) soluble fractions prepared from methanol extracts of processed and unprocessed rooibos and honeybush teas on tumour promotion in mouse skin, using green tea as reference.

MATERIALS AND METHODS

Chemicals. 7,12-dimethylbenz[a]anthracene (DMBA) and 12-O-tetra-decanoylphorbol-13-acetate (TPA), butylated hydroxytoluene (BHT), 2-thiobarbitric acid (TBA), ethylene diamine tetra-acetic acid disodium salt (EDTA), gallic acid, (+)-catechin, quercetin, mangiferin, rutin, p-dimethylaminocinnamaldehyde (DAC) and Folin's reagent, were purchased from Sigma Chemical Co. (Cape Town, South Africa). Hesperidin and hesperetin were obtained from Aldrich Chemical Co (Cape Town, South Africa). Orientin, iso-orientin, vitexin, iso-vitexin, luteolin, chrysoeriol and iso-quercitrin were purchased from Extrasynthese (Genay, France). Aspalathin and nothofagin, isolated from unprocessed/"green" rooibos to a purity of >95%, were supplied by Ms P Snijman (PROMEC Unit, MRC, South Africa). All other chemicals used were of analytical grade.

Animals. Six-week old female ICR mice were obtained from the Animal Unit, Medical School, University of Cape Town (South Africa) and kept in the Animal Unit of the University of Stellenbosch, (Tygerberg, South Africa) for at least one week prior to commencement of the experiment. Mice had free access to pelleted murine chow (Epol LTD, Johannesburg, South Africa) and water while kept on a 12 hr photoperiod with optimum air changes per hour and a constant room temperature of 21 ^{0}C.

Plant material. Processed (oxidised) and unprocessed ("green"/unoxidised) rooibos and honeybush teas, part of the indigenous "fynbos" flora in certain coastal and mountainous regions of the South Western Cape Province (South Africa), were obtained from ARC Infruitec-Nietvoorbij, (Agriculture Research Council), Stellenbosch,

South Africa. The green tea (*Camellia sinensis* var. *sinensis*), imported from China, was a gift from Vital Health Foods, Kuils River, South Africa.

Preparation of the ethanol/acetone soluble (E/A) extracts. Polyphenolic extracts of both processed and unprocessed rooibos and honeybush and Chinese green teas were prepared by extracting (3 times) grounded plant material with chloroform (3% m/v) by vigorous stirring in an Erlenmeyer flask and filtering through Whatman no 3 filter paper. The residual plant material was exhaustively extracted overnight with methanol as described above, filtered, the solvent evaporated under reduced pressure at 40 ^{0}C in a Rotavapor and the residue stored in an airtight container at 4 ^{0}C in the dark. The E/A (1:1 v/v) soluble fraction of the different tea preparations was prepared by reconstituting 30 mg of the methanol extract in 1 mL absolute ethanol and acetone (1:1) mixture.

Soluble solids, total polyphenolic, flavanol/proanthocyanidin and flavonol/flavone content of the E/A soluble fractions. The solid content of each E/A soluble tea fraction was determined gravimetrically (4 repetitions) after drying 1 mL aliquots at 110 ^{0}C for 12 hr. The Folin-Ciocalteau method with gallic acid as standard was used to determine the total polyphenol (TP) content [20]. The flavanol/proanthocyanindin and flavonol/flavone contents were determined colorimetrically (640 nm) using DAC with (+)-catechin as standard [21] and spectrophotometrically (360 nm) using quercetin as standard [22], respectively. The results were expressed as mg catechin or mg quercetin equivalents/mg soluble solids. The possible contributions of hesperidin, mangiferin and aspalathin to the flavanol/proanthocyanidin content were also determined spectrophotometrically using reactivity with DAC as a measure.

HPLC quantification of the major flavonoids in the E/A soluble herbal tea fractions. Dried E/A fractions of rooibos and honeybush were dissolved in methanol and filtered through a Magna Nylon 13 mm 0.45 μm filter prior to HPLC analysis. The honeybush extracts were analysed for mangiferin, hesperidin and hesperetin according to the method of Joubert et al. [15]. HPLC analysis were conducted on a LaChrom2000 system comprising of an L-7400 UV detector, L-7100 pump, Rheodyne 7725i injection valve and D-7000 HPLC system manager and interface using a Phenomenex Synergy Max-RP C12 80A (4 μm, 150 x 4.6 mm I.D.) column while quantification was done at 280 nm. The rooibos extracts were analysed for the dihydrochalcones, aspalathin and nothofagin and the flavones, orientin, iso-orientin, vitexin, iso-vitexin, luteolin,

chrysoeriol and flavonols quercetin, iso-quercitrin and rutin. Rutin co-eluted with iso-quercitrin and the quantification was expressed as quercetin equivalents. A Waters LC Module 1 Plus system, 2996 diode array detector and Millenium Version 3.2 for system control and data acquisition were used. Separation was conducted on a Merck Lichrosphere 100 RP-18 (5 μm, 250 x 4 mm I.D.) column. Quantification was conducted at 288 nm (dihydrochalcones) and 255 nm (flavones and flavonols) according to the method of Joubert [23] with a modification to the initial gradient. A calibration curve for each flavonoid was prepared.

Thiobarbituric acid reacting substances (TBARS) determinations. The formation of TBARS, measured as malondialdehyde (MDA), was used to determine the protective effect of the E/A soluble fractions (0.01% dissolved in DMSO) against lipid peroxidation utilising a rat liver microsomal (1 mg protein/mL) preparation. Lipid peroxidation was determined using a modified method of Yen and Hsieh [24] in the presence of Fe^{2+} and absence of hydrogen peroxide. Results were expressed as nmol MDA per mg protein using the millimolar extinction coefficient of 153 [25]. The microsomal fraction was prepared from an S-9 liver homogenate by Sepharose 2B column chromatography in Tris buffer (50 mM, pH 7.4) containing KCl (150 mM) as previously described [26]. The microsomes were stored at –80 ^{0}C until used. Protein determination was performed according to the method of Kaushal and Barnes [27] using bovine serum albumin as standard.

Effect on tumour promotion. Eighty mice were randomly divided into 8 treatment groups of 10 animals each and caged separately. Prior to the study the dorsal side of the skin was treated with a depilatory cream (No Hair; Adcock Ingram, South Africa). Mice were treated with a single topical application of DMBA (200 nmol) in acetone (100 uL) as tumour initiator followed by the cancer promoter, TPA (5 nmol; 100 uL), one week later. The protective effect of the E/A tea fractions was monitored by topical application (100 uL) 30 min prior to TPA treatment. Positive control (DMBA/TPA) mice were treated with the carrier solvent in a similar manner (acetone/ethanol). Two experimental control groups were included consisting of DMBA/acetone and acetone/TPA treatments. The treatments were repeated twice weekly for 20 weeks and the number of skin tumours was recorded weekly. The final tumour yield and tumour volume, using tumour height and internal diameter measures, were determined upon

termination by CO_2 asphyxiation. Details of the experimental outlay are summarised in figure 1. The experiment was repeated once.

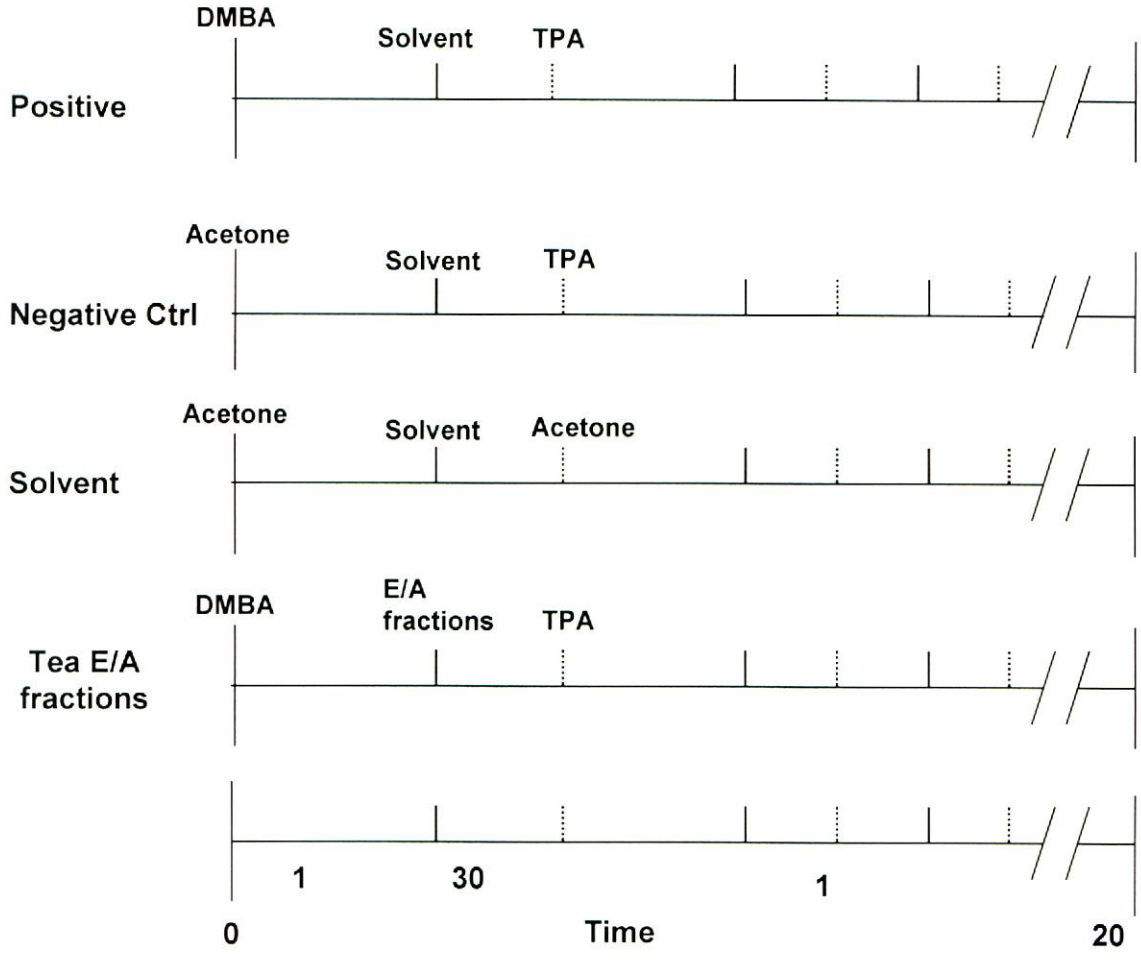


Fig. 1. Experimental protocol of the various treatment regimens used in the two-stage mouse skin cancer model. Mice were treated with a single dose of DMBA (200 nmol) or the control solvent (acetone). Modulation of cancer promotion was conducted by the application of the tea ethanol/acetone (E/A) polyphenolic fractions 30 minutes prior to TPA (5 nmol) twice weekly for 20 weeks.

Statistical analyses

An Analysis of Variance (ANOVA) was performed on the data using Proc GLM in SAS. Where equal group variances were present, the F-test was used to test for group differences, using the parametric Tukey-test to indicate which specific groups differed significantly. Otherwise the Welch test was used where groups had unequal variances. Student's T-test was used to test for pair wise group differences.

RESULTS

No noticeable clinical signs of illness e.g. weight loss, diarrhoea, rhinitis, alopecia were detected in any of the groups as a result of the topical application of the different treatments.

Soluble solids, total polyphenolic and flavonoid content of the E/A soluble fractions.

No significant ($P > 0.05$) differences were noticed in the soluble solid content of the various fractions. The TP content of the E/A soluble fractions of the processed herbal teas (13-24%) were significantly ($P < 0.001$) to marginally ($P < 0.1$) lower when compared to their unprocessed counterparts (31-55%) as well as the green tea (56%) (Table I). The green tea E/A fraction had a similar TP content to that of the unprocessed rooibos. The E/A fractions of the unprocessed herbal teas had a similar flavanol/proanthocyanidin content while their processed counterparts had a significantly lower ($P < 0.001$) content, with the processed honeybush fraction containing the lowest amount ($P < 0.001$). When expressed as a percentage of the TP content, the green tea and unprocessed honeybush tea E/A fractions contain up to 90% flavanols/proanthocyanidins, followed by unprocessed rooibos (47%), processed rooibos (22%) and processed honeybush (14%). The flavonol/flavone content of the processed and unprocessed rooibos E/A fraction was significantly ($P < 0.001$) higher than the honeybush and green tea fractions. The unprocessed honeybush E/A fraction contained a marginally ($P < 0.1$) higher flavonol/flavone content when compared with green and processed honeybush fractions. When considering the flavonol/flavone content as a percentage of the TP content the E/A fractions of the unprocessed herbal teas (~ 4.7%), exhibited the lowest amounts when compared to the processed counterparts (10 -15 %) which were still significantly ($P < 0.001$) higher than the green tea E/A fraction (1.8%).

None of the major flavonoid constituents of the E/A fractions prepared from honeybush and rooibos, aspalathin, mangiferin and hesperidin showed a positive reaction with DAC when the flavanols were determined.

Quantification of selective flavonoids of herbal E/A fractions. Aspalathin and nothofagin comprised the major flavonoids in the E/A fraction of the unprocessed rooibos (Table II). The flavone oxidised products of aspalathin, orientin and iso-orientin

and those derived from nothofagin, vitexin and iso-vitexin were also detected. Rutin and isoquercitrin, that co-eluted, were present while only trace amounts of quercetin, luteolin and chrysoeriol were detected. A similar pattern was noticed in the E/A fraction from the processed rooibos with the exception that aspalathin and nothofagin were reduced while their oxidised analogues were increased. Rutin/isoquercitrin, quercetin, luteolin and chrysoeriol could also be detected. Mangiferin and hesperidin were the major flavonoid constituents detected in the unprocessed and processed honeybush E/A fractions. As noticed for rooibos tea, the honeybush flavonoids were markedly reduced as a result of tea processing.

Based on the volume of each fraction applied to mouse skin, aspalathin was the major flavonoid in the unprocessed rooibos E/A fraction whilst iso-orientin, orientin and aspalathin represented the major flavonoids when administering the processed rooibos fraction. Mangiferin and hesperidin were the major compounds in both the unprocessed and processed honeybush fractions (Table II).

Inhibition of microsomal lipid peroxidation. The unprocessed herbal tea fractions exhibited a higher protective effect against lipid peroxidation when compared to their processed counterparts (Table I). Of the herbal teas unprocessed rooibos exhibited the highest protective effect while processed rooibos and unprocessed honeybush showed a similar but lower protection. The processed honeybush fraction was the least effective against the inhibition of lipid peroxidation with the green tea fraction exhibiting the highest ($P < 0.05$) protective effect of all the teas fractions.

Inhibition of tumour promotion. None of the mice in the negative control groups (DMBA/solvent and solvent/TPA) developed skin tumours. The positive control mice (DMBA/TPA) developed a mean number of 5.3 skin tumours per mouse (Fig. 2A) with a tumour incidence of 88.3% (Fig. 2B). No tumours developed in the mice treated with the green tea E/A fraction. Topical application of the various herbal tea E/A fractions significantly ($P < 0.001$) protected against tumour promotion by TPA (Fig. 2A). The mean number of tumours per mouse was significantly reduced by unprocessed rooibos (1.1) followed by processed rooibos (0.7), unprocessed (0.3) and processed (0.2) honeybush (Fig. 2B) fractions. Unprocessed honeybush exhibited the highest decrease (90%) in the % tumour bearing mice, followed by processed honeybush (84.2%), processed rooibos (75%) and unprocessed rooibos (60%) fractions.

Table I. Soluble solids, total polyphenolic, flavanol/proanthocyanidins and flavonol/flavone contents of the ethanol/acetone tea extracts topically applied to mouse skin. The inhibitory effect on of lipid peroxidation was monitored in rat liver microsomes (1mg protein/mL) in the present of Fe^{2+}

Tea extracts	Soluble solids (mg/100uL)	TP content (mg Gallic acid equivalents/mg soluble solids)*	Flavanol/ proanthocyanidin content (mg Catechin equivalents/mg soluble solids)**	Flavanol/ proanthocyanidin content (expressed as a % of TP)	Flavonol/flavone content (mg Quercetin equivalents/mg soluble solids)#	Flavonol/flavone content (expressed as a % of TP)	$TBARS (nmol MDA/ mg protein)
Rp	2.24 ± 0.36a	0.24 ± 0.03a (24%)	0.05 ± 0.001a (5.4%)	21.90 ± 0.05a	0.037 ± 0.010a (3.7%)	15.07 ± 4.57a	1.40 ± 0.44a
Rg	2.29 ± 0.20a	0.55 ± 0.08b (55%)	0.26 ± 0.004b (26%)	46.46 ± 0.12b	0.026 ± 0.002a (2.6%)	4.68 ± 0.31b	0.37 ± 0.10b
Hp	2.16 ± 0.46a	0.13 ± 0.02(a) (13%)	0.02 ± 0.001c (2%)	13.62 ± 0.01c	0.014 ± 0.008b (1.4%)	10.52 ± 6.37a	3.50 ± 0.43c
Hg	2.07± 0.42a	0.31 ± 0.04a (31%)	0.28 ± 0.001b (28%)	91.04 ± 0.09d	0.015 ± 0.003 (b) (1.5%)	4.84 ± 1.12b	1.47 ± 0.31a
Gr	2.57± 0.41a	0.56 ± 0.05b (56%)	0.51 ± 0.002d (51%)	91.50 ± 0.16d	0.010 ± 0.002b (1%)	1.78 ± 0.4c	0.04 ± 0.01b
Ctrl	-	-	-	-	-	-	4.01± 0.10d

$TBARS assay conducted using 0.01% (w/v) of the E/A polyphenolic fraction dissolved in DMSO. Values are means ± STD of 17-20 determinations, except for soluble solids (n=4). Means in columns followed by the same letter are not significantly different. If the letters differ then $P<0.001$. Letters in parenthesis then $P<0.1$. *, **, # values expressed as a % of the soluble solids. Abbreviations: Rp = processed rooibos, Rg = unprocessed rooibos, Hp = processed honeybush, Hg = unprocessed honeybush and Gr = green tea. TP = total polyphenols.

Table II. Quantitative amount of flavonoids applied to the mice skin using processed and unprocessed herbal ethanol/acetone tea fractions

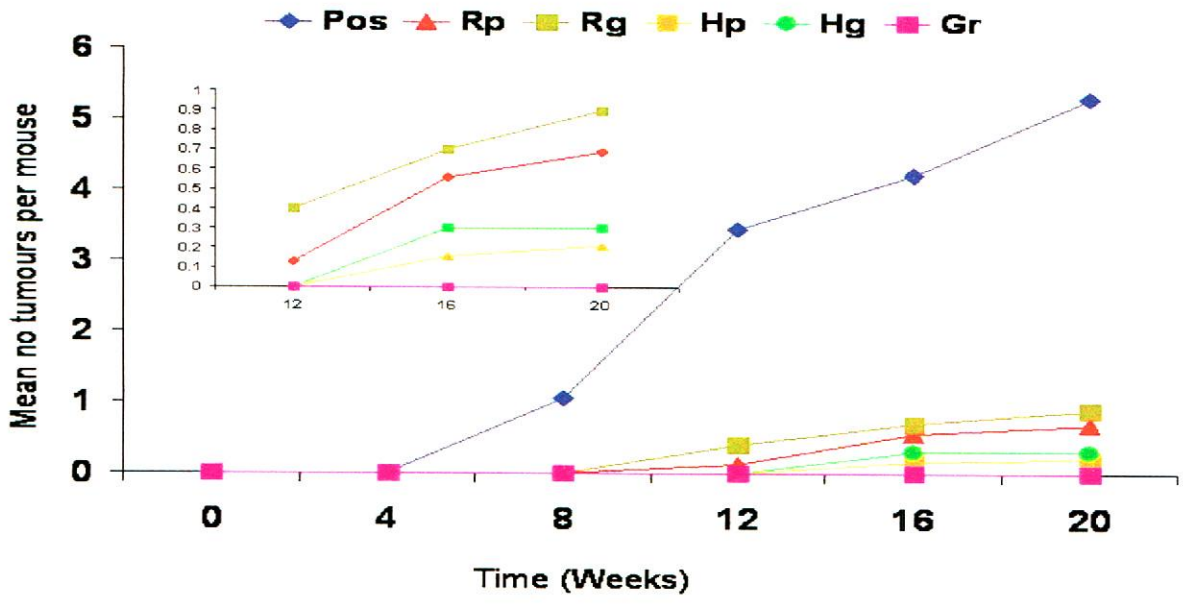
Polyphenols	E/A fractions			
	Processed		Unprocessed	
	% soluble solids	µg/100 µl	% soluble solids	µg/100 µl
Rooibos constituents				
Aspalathin	2.12 ± 0.02	63.45 ± 0.62	5.67 ± 0.06	226.82 ± 2.31
Nothofagin	0.24 ± 0.01	7.36 ± 0.03	0.81 ± 0.01	32.47 ± 0.15
Orientin	2.43 ± 0.01	72.80 ± 0.09	0.54 ± 0.01	21.68 ± 0.10
Iso-orientin	3.08 ± 0.01	92.33 ± 0.03	0.65 ± 0.01	26.15 ± 0.03
Vitexin	0.37 ± 0.01	11.24 ± 0.02	0.06 ± 0.01	2.49 ± 0.04
Iso-vitexin	0.94 ± 0.04	28.29 ± 0.12	0.27 ± 0.01	10.76 ± 0.04
Rutin/Iso-quercitrin	0.74 ± 0.01	22.09 ± 0.17	0.31 ± 0.01	12.24 ± 0.07
Quercetin	0.13 ± 0.01	3.83 ± 0.25	Trace	Trace
Luteolin	0.11 ± 0.01	3.19 ± 0.35	Trace	trace
Chrysoeriol	0.10 ± 0.01	2.91 ± 0.002	Trace	trace
Honeybush constituents				
Mangiferin	3.74 ± 0.01	112.19 ± 0.52	5.61 ± 0.08	168.42 ± 2.37
Hesperidin	3.69 ± 0.31	110.69 ± 9.32	4.14 ± 0.15	124.26 ± 4.42
Hesperetin	trace	Trace	0.41 ± 0.01	12.24 ± 0.01

The values in columns represent the mean ± standard deviation of 2-3 determinations.

When considering the mean tumour volume/mouse a similar trend was observed. The DMBA-initiated mice receiving TPA promotion had a mean tumour volume (mm^3) per mouse of 80.4 ± 158.6, compared to 34.7 ± 130.4 for processed rooibos, 27 ± 55.6 for unprocessed rooibos, 11.3 ± 35.7 for unprocessed honeybush and 1.8 ± 5.7 for processed honeybush.

The herbal tea fractions not only decreased the tumour volume and the mean number of tumours per mouse (Fig. 2A) but also delayed tumour development (Fig. 2A, B). The first tumour in the DMBA/TPA treated mice appeared at 4 weeks, 12 weeks when treated with the processed and unprocessed rooibos and 16 weeks when treated with the processed and unprocessed honeybush E/A fractions.

A



B

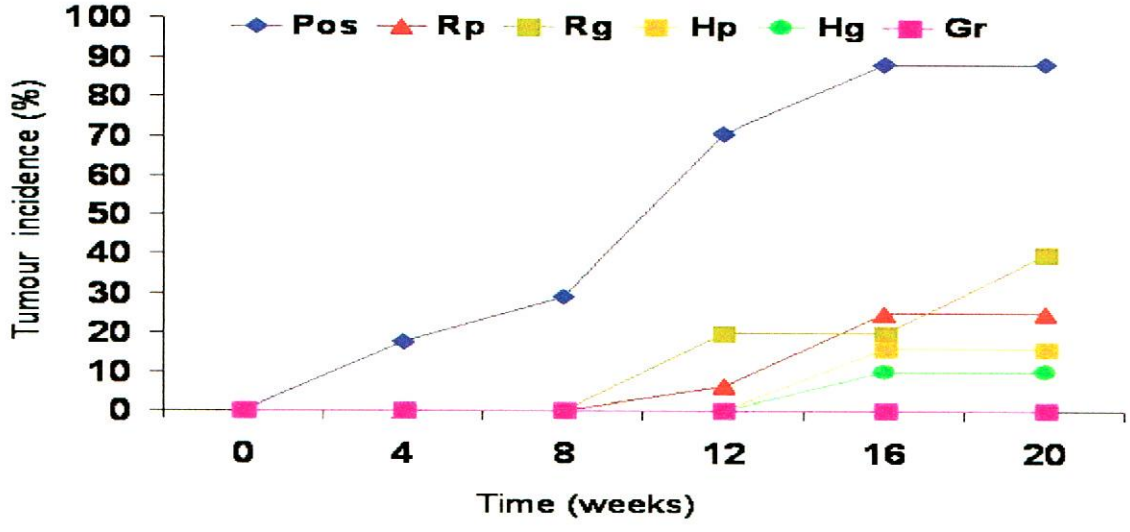


Fig. 2. Inhibitory effect of topical application of various tea E/A polyphenolic fractions on TPA-induced tumour promotion in mouse skin. The mean number of tumours per mouse (A) and the percentage mice with tumours (B) is plotted as a function of the treatment period (weeks). The fractions include Rp = processed rooibos, Rg = unprocessed rooibos, Hp = processed honeybush, Hg = unprocessed honeybush and Gr = green tea. The number of animals per group = 17-20. (Fig 2a insert – skin tumour incidence in mice treated with the herbal teas extracts between 12 to 20 weeks).

DISCUSSION

The mechanisms involved in mouse skin tumour promotion have not been fully characterised but events such as hyperplasia, inflammation and production of reactive oxygen species (ROS) amongst others, have been shown to be important [12,28,29]. Pre-treatment of murine skin with natural antioxidants suppressed the oxidative stress activity of ornithine decarboxylase (ODC), cell proliferation and ultimately inhibited skin tumour promotion [30,31]. Topical application of green and black tea polyphenols prior to TPA administration protected against the onset and subsequent development of skin tumours [7,13,32]. Polyphenolic fractions of green tea exhibited antioxidant activity towards hydrogen peroxide and superoxide anion radicals in mouse hepatocytes and human keratinocytes [33,34]. Thus, compounds exhibiting antioxidant and/or anti-inflammatory activities are expected to be effective anti-tumour promoting agents [35].

The present study confirms that a polyphenolic fraction of green tea, containing mainly flavanol/proanthocyanidin constituents, inhibits the formation of mouse skin tumours. Evidence is also provided for the first time, that E/A soluble fractions prepared from unprocessed and processed rooibos and honeybush methanol extracts protect against TPA-induced tumour promotion in mouse skin. A significant ($P < 0.05$) decrease in tumour incidence, a marked reduction in tumour volume and a delay in the onset of tumour development were shown in the present study. Previous studies showed aqueous extracts of the herbal teas reduce oxidative stress, as well as enhance the antioxidant capacity in rat liver after chronic exposure [18,19]. Aqueous extracts of processed rooibos tea also significantly inhibited the activity of ODC in fibroblasts and myoblasts in tissue culture which were ascribed to the potent radical scavenging activity of rooibos tea extract [36]. Data from these studies and the present study suggest that the antioxidant activity of the different tea E/A fractions are likely to be involved in the reduction of ROS induced by TPA thereby inhibiting tumour promotion.

The green tea fraction with the highest TP content, exhibited the best protection against lipid peroxidation and tumour promotion. Inhibition of lipid peroxidation by the herbal tea fractions are in accordance with previous data indicating a relationship between the polyphenolic content of aqueous rooibos and honeybush tea extracts and their antioxidant potency [37-41]. However, no relationship existed between the TP content and the inhibition of lipid peroxidation and the protection against tumour promotion in the present study. It seems that differences in and/or the relative

concentrations of flavonoid subgroups constituting the TP of the herbal E/A fractions, will determine their protective potency against tumour promotion.

When considering flavonoid subgroups, the flavanol/proanthocyanidin content, expressed as a percentage of the TP content, both green tea and unprocessed honeybush tea fractions exhibited similar levels (approximately 90%). This would imply that the flavanol content as well as the type of flavanols and/or flavanol-like compounds, such as the proanthocyanidins and polymeric tea tannins could also play an important role in the inhibitory effect on tumour promotion. In this regard, the E/A fractions from green and unprocessed honeybush showed the strongest reaction when utilising the butanol/HCl method [42] to determine the presence of proanthocyanidins (unpublished data). The proanthocyanidins, therefore, are likely to make a major contribution towards the flavanol content of the green and unprocessed honeybush fractions when compared to the other herbal tea E/A fractions. The proanthocyanidins from grape seed oil have been shown to inhibit photo- and chemically- induced skin carcinogenesis in mice [43,44]. Of interest is that the major flavonoids determined in the honeybush tea fractions, hesperidin and mangiferin, did not contribute to the flavanol content. A previous study also reported on the lack of hesperidin to react with DAC during the colourimetric determination of flavanols [45].

The flavonol/flavone content of both the unprocessed honeybush and rooibos fractions contained approximately 5% of the TP content. As the unprocessed honeybush fraction exhibited the highest protective effect it would imply that the flavonol/flavones play a secondary role to the flavanols/proanthocyanidins. This is further supported by the fact that the E/A fraction of green tea exhibiting the highest protective effect also contain the lowest flavonol/flavone content (~2%). A higher flavonol/flavone and lower flavanol/proanthocyanidins content exhibited intermediate effects against tumour promotion, as in the case of the processed rooibos and honeybush fractions. As described for honeybush tea, the proanthocyanidins of rooibos tea could also be involved, as tannins of unprocessed rooibos is comprised of epicatechin and catechin-extending units [46].

The polyphenolic composition of the herbal teas differs from one another [47-50] and from green and black teas [51,52]. Purified green tea flavanols, epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epigallocatechin (EGC) and epicatechin (EC) inhibited TPA-induced inflammation of mouse ears [11]. The flavonol, quercetin,

possessing reactive oxygen scavenging activity, also inhibits skin tumour promotion and certain biochemical effects associated with skin tumour promotion [11,53]. The antioxidant properties of the purified polyphenolic compounds from rooibos have been well characterised in different *in vitro* radical scavenging and lipid peroxidation assays [37-39,41,54]. Aspalathin exhibits a similar antioxidant activity to EGCG [55]. At present no data is available on the inhibitory effects on cancer promotion of the rooibos flavonoids aspalathin, nothofagain, orientin and iso-orientin, which constituted the major flavanoids in the processed and unprocessed rooibos fractions, applied to the mouse skin. Rutin, quercetin and luteolin, present to a far lesser extent in the processed rooibos fraction are known to exhibit anti-inflammatory activity [56]. The honeybush flavonoids, hesperidin and hesperetin also possess anti-inflammatory activity [57,58], while mangiferin is the major antioxidant of honeybush [59]. Both the unprocessed and processed honeybush fractions contained high amounts of hesperidin, which together with mangiferin constitutes the major flavonoids applied to mouse skin. The better protective effect by the unprocessed and processed honeybush E/A fractions against tumour promotion, when compared to rooibos, could in part be attributed to the anti-inflammatory activity of these compounds. However, the protective properties against skin tumour promotion by hesperedin, mangiferin and other minor honeybush tea flavonoids, including glycosylated flavonols, isoflavones, flavanones [50] and the coumestans [49] still need to be elucidated.

As the polyphenolic content of the honeybush E/A fractions only constitute 13 to 23% of the soluble solids and 24 to 50% for rooibos, the non-flavonoid components could also play a major contributing role in the inhibition of tumour promotion. Subsequent studies need to be conducted to elucidate whether the herbal polyphenolic fractions and/or specific flavonoids could modulate biological events involved during TPA-induced tumour promotion in mouse skin.

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CHAPTER 8

Summary and Conclusion

Summary

Theoretically cancer is a preventable disease, but in practice, prevention by avoiding exposure cannot be achieved and additional approaches are needed. One such approach is an increased intake of chemopreventive compounds that is expected to interfere with the initiation, promotion or progression of carcinogenesis. Chemopreventive compounds are largely categorized as enzyme modulators, antioxidants, inhibitors of carcinogen-adduct formation, inhibitors of oncogene activation, modulators of signaling cascades associated with carcinogenesis, to name a few. Although considerable advances have been made in the field of chemoprevention, more research is needed to establish this as an additional approach to alleviate the burden of cancer. The past 10 years yielded important information from across the world concerning the positive impact of non-nutritive components of plant origin like teas on human health. Special attention has been paid to the development and availability of laboratory methods and has led to an explosion in the neutraceutical industry utilizing herbal plant extracts in combating human disease.

Studies with green and black teas have led the way in utilizing tea infusions as possible chemopreventive agents against cancer development in humans. Considerable work has been carried out on both green and black teas and their polyphenolic components that provide valuable information regarding the possible protection against carcinogenesis *in vivo*. The protective role of green tea against human cancers is evident from the number of epidemiological studies, while the data on black tea are limited. The antioxidant activities of green tea are well established, and the ability to induce various antioxidant enzymes, inhibit of the metabolic activation of carcinogens, induce certain P450s and phase II drug metabolizing enzymes, be antimutagenic in various microbial systems and mammalian cells and inhibit tumour promotion in mouse skin, lung, oesophagus, stomach, liver and colon have been previously reported. The two most common South African herbal plants used for the preparation of traditional health drinks are rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* species). Having been used by local communities for centuries, anecdotal evidence provides important information regarding their possible use against certain human ailments. The herbal teas not only differ from one another but also from green and black teas with respect to their polyphenolic constituents while they are naturally caffeine free with low tannin content. The present study was conducted to investigate the possible cancer modulating properties of the herbal teas and included short term *in vitro*

antimutagenicity, *ex vivo* antimutagenicity, *in vivo* modulation of drug metabolizing enzymes and oxidative status of cells, and tumour promotion in rodent models.

The *in vitro* antimutagenic activity of aqueous extracts of processed ("fermented") and unprocessed ("unfermented") rooibos and honeybush teas, was evaluated in the *Salmonella* strains TA 98, TA 100 and TA 102 using five different mutagens with diverse chemical structures and mode of action, in the presence and absence of S9. All the herbal tea extracts significantly inhibited the mutagenicity of the metabolic activated mutagens, 2-AAF and AFB_1. However, the inhibitory effect on the direct acting mutagens, MMS, CHP and H_2O_2 using tester strain TA 102, designed to detect oxidative damage, was far less pronounced. During tea processing ("oxidation"), the polyphenolic content decreases substantially and in general this was reflected in a lower antimutagenic activity dependant on the mutagen used. Variations of the double layer *Salmonella* mutagenicity assay further elucidated the possible mechanisms of protection involved in the antimutagenic activities of the herbal teas. With respect to the metabolic activated mutagen, 2-AAF, a higher degree of protection was obtained when the herbal teas were incubated with the mutagen and activating (S9) mixture in a separate layer to that of the bacterial strain, TA 98 as opposed to when the herbal teas were incubated with the bacterial strain. These results suggested that two mechanisms are involved in the *in vitro* antimutagenic activity of the herbal tea extracts against carcinogens requiring metabolic activation. The tea components may (i) interfere with the enzyme system (P450) catalysing the metabolic activation of the carcinogens and thus impede the production of the activated intermediates and/or (ii) may directly interact with the pro-carcinogen and/or reactive intermediate resulting in the prevention of mutagenesis. The mild and/or lack of protection against mutagenesis induced by the direct acting/or oxidative mutagens, suggested that an induction of antioxidant protective mechanisms *in vivo* may be required to protect against these mutagens. Both the processed as well as unprocessed rooibos tea extracts showed a similar inhibition against H_2O_2-induced mutagenesis, despite the fact that the total polyphenols were significantly lower in the processed herbal tea preparations. No relationship seems to exist between the antioxidant activity of the herbal teas and the antimutagenic or lack of antimutagenic activity against direct acting mutagens *in vitro*.

Results from this study indicated that:

1. the mechanism of protection by the herbal teas against metabolic activated and direct acting mutagens differs, while the type of mutagen used also plays a role.
2. rooibos and honeybush teas may not only be a good dietary source of antioxidants (as previously suggested in the literature) but also may protect against mutagenesis *in vivo.*

The next study was conducted to determine the effect on hepatic drug metabolizing enzymes and oxidative status by rooibos and honeybush teas in rats consuming aqueous extracts at concentrations customarily used for human consumption. The balance between phase I and phase II enzymes is critical in the subsequent production of carcinogenic/mutagenic metabolites that will interact with the cellular components. The level and/or activity of these enzymes plays an important role in the susceptibility of an individual for developing cancer. Rooibos and honeybush teas significantly enhanced the activity of the cytosolic phase II enzyme, GST-α, while the unprocessed herbal teas also induced the activity of the microsomal phase II enzyme UDP-GT. The induction of UDP-GT activity in microsomes has been suggested as a major phase II carcinogen deactivating mechanism by green tea. None of the herbal teas displayed any effect on the concentration of the phase I activating enzyme, P450. The polyphenolic components of the herbal teas also modulated the cellular oxidative status in the liver of rats. Both herbal teas significantly reduced the level of hepatic GSSG, as well as markedly increasing the level of GSH, resulting in an increased GSH:GSSG ratio. An increased ratio is indicative of a reduced oxidative stress or an increased antioxidant capacity in the liver cells, thereby lowering the susceptibility to undergo oxidative damage. The stabilization of GSH in the liver may result in an increase in the endogenous detoxification capacity, as GSH is known to either bind directly or enzymatically via GST to reactive genotoxic metabolites, thereby preventing DNA damage. The induction of phase II hepatic drug metabolizing enzymes and the increased antioxidant status of the liver by rooibos and honeybush teas provides a promising tool for the protection against adverse effects related to mutagenesis and oxidative damage. Rats also demonstrated no adverse effects on the body weight gain, relative liver weights and serum enzymes related to liver and kidneys function. The lack of toxicity confirms what has been noticed in man consuming the herbal teas for more than 3 centuries.

Following this study, it was imperative to establish whether consumption of the herbal teas may protect against mutagenicity of 2-AAF and AFB_1 in the *Salmonella* assay conducted under *ex vivo* conditions. Hepatic cytosolic fractions of rats consuming unprocessed herbal teas and to a certain extent the processed herbal teas, protected against 2-AAF-induced mutagenesis using tester strain TA 98 and Aroclor 1254-induced microsomes. Both processed as well as unprocessed herbal teas also protected against the mutagenicity induced by AFB_1 using tester strain TA 100. Hepatic microsomal fractions prepared from the rats consuming the processed and unprocessed rooibos and unprocessed honeybush teas reduced the activation of AFB_1 while no protection was observed against 2-AAF-induced mutagenesis. Thus, in the case of AFB_1, oral administration of rooibos and honeybush influenced both the microsomal activation as well as the cytosolic protection against mutagenesis whereas with 2-AAF, only the cytosolic components contributed to the protective effect. Differences in the *ex vivo* modulation of mutagenesis by these carcinogens could be ascribed to differences in the metabolic pathways resulting in the formation of the ultimate active metabolite. This study confirmed that the *in vitro* antimutagenic activity of the herbal teas against AFB_1- and 2-AAF-induced mutagenesis is likely to prevail under *in vivo* conditions. These results also provided the first evidence that components of rooibos and honeybush teas involved in the *ex vivo* modulation of 2-AAF and AFB_1 metabolism, are absorbed from the gut and are available to exert their protective effects.

With the established *in vitro* and *ex vivo* antimutagenic properties, the *in vivo* modulation of drug metabolizing enzymes and oxidative status in the liver by the herbal teas and the cancer modulating properties were investigated. As the role of dietary intervention during the different stages of carcinogenesis, such as cancer promotion is complex, short-term carcinogenesis models in experimental animals continue to play an important role in identifying potential cancer modulating compounds and validating the mechanisms involved. Numerous studies are being conducted to investigate the role of chemopreventive agents as inhibitors of tumour promotion. The possible chemoprotective properties of aqueous extracts of processed and unprocessed rooibos and honeybush teas against the cancer promoting activity of FB_1 in rat liver using DEN as initiator and the induction of hepatic preneoplastic foci as endpoint, was therefore elucidated. Consumption of the herbal teas arrested the proliferation of $GSTP^+$ cells in the presence of the cancer promoter, FB_1, as the relative number of smaller foci was

significantly increased while the larger foci was decreased. Consumption of the unprocessed herbal teas also reduced the total number of $GSTP^+$ foci presumably by increasing the hepatic toxicity and the subsequent apoptotic effects of FB_1 thereby inhibiting the growth of the preneoplastic lesions. A possible mechanism involved in this enhanced hepatotoxicity of FB_1 could be the mobilization of iron in the liver, resulting in a prooxidant effect when interacting with the polyphenolic compounds of the herbal teas. In this regard the processed herbal teas, known to have a lower antioxidant capacity, exhibited a higher protection against FB_1-induced lipid peroxidation which was also associated with a reduced accumulation of GSSG, an indicator of increased hepatic stress. Unprocessed rooibos, green and black teas, known to have a high antioxidant capacity are less effective in counteracting lipid peroxidation effected by FB_1, presumably due to prooxidant effects of their polyphenolic constituents. Due to the complexity of these interactions no conclusive evidence with respect to the involvement of antioxidant activities of the herbal teas and the decreased number and/or delayed development of $GSTP^+$ foci in the liver could be demonstrated. Varying effects on the ORAC, GSH levels and the inhibition of lipid peroxidation were noticed which could be ascribed to the differences in polyphenolic constituents among the teas and their interaction with FB_1-induced hepatotoxic effects. Other features, affecting cell regulatory processes, may be involved and still need to be elucidated.

The chemopeventive properties of the herbal tea extracts were further investigated by monitoring the protective effects of polyphenolic extracts in the two stage skin carcinogenesis model. It is known that a variety of plant phenolic compounds exhibit chemoprotective properties by disrupting the different stages of multi step skin carcinogenesis, especially tumour promotion. The present study indicated that polyphenolic fractions prepared from methanol extracts of processed and unprocessed rooibos and honeybush teas effectively reduced tumour formation in mouse skin initiated with DMBA by interfering with TPA promotion. The protected effect was shown by a decrease in tumour incidence, a reduction in tumour volume as well as a delay in the onset of tumour development. The processed herbal tea fractions exhibited a similar inhibition while the unprocessed honeybush tea showed a higher inhibition potential than the unprocessed rooibos fraction. Elucidation of the flavonoid sub groups of the herbal teas, suggested that the flavanol content as well as the type of flavanol and/or flavanol like compounds, such as the proanthocyanidins, could play an important role in the inhibitory effect. This hypothesis was supported by the finding that green tea, with a

high flavanol/proanthocyanidin content, was the most effective inhibitor of TPA-induced tumour promotion. Fractions with a lower flavanol/proanthocyanidin content and higher flavonol/flavone content, as found in the processed and unprocessed rooibos fractions, exhibited an intermediate effect against cancer promotion. The higher protective effect by the unprocessed and processed honeybush fractions when compared to the rooibos fractions could in part be attributed to the anti-inflammatory activity of hesperedin, which together with mangiferin, constitutes the major flavonoids in the honeybush fractions applied to the skin. Chronic inflammation in mouse skin facilitates growth of initiated cells and their subsequent progression into malignancy.

Quantification of the major flavonoids in the aqueous and methanolic extracts showed that the dihydrochalcones, aspalathin and nothofagin comprised the main flavonoids in the unprocessed rooibos extracts, while their flavone counterparts, orientin, iso-orientin, vitexin and iso-vitexin and the flavonol glycosides, rutin/isoquercitrin constituted the major flavonoids in the processed rooibos. Aspalathin and nothofagin levels were reduced during processing, resulting in an increased intake/application of iso-orientin and orientin relatively to aspalathin. The main flavonoids detected in the unprocessed and processed honeybush tea extracts were the xanthone, mangiferin and the flavanone, hesperidin, while small amounts of hesperetin, the aglycoside, were also detected in the unprocessed tea. Further characterisation and quantification of other flavonoids in honeybush tea extracts including the glycosylated flavonols, isoflavones, flavanones and the coumestans is currently in progress

The antimutagenic and anticarcinogenic properties of green and black teas are well known and were confirmed in the present study. Rooibos and honeybush teas exhibit similar properties with respect to the modulation of drug metabolising enzymes and the oxidative status than green and black teas in the liver. However, variables such as the rat species, amount of tea consumed, duration of tea treatment, route of administration, and substrates used in the enzymes assays should be taken into account when comparing the different studies. In addition, the flavonoid constituents and content of the tea preparations and the different mechanisms involved underlying the complex processes of carcinogenesis could be responsible for the differences noticed between the different teas in the present study. This becomes evident when comparing the chemopreventive properties of the different teas with respect to their effect on drug metabolizing enzymes and against tumour promotion in the liver and

mouse skin. However, this study indicated that the herbal teas exhibit similar cancer modulating properties than the green and black teas.

Conclusion

Humans are constantly seeking to advance their health and alleviate various ailments with herbal remedies. An integrated approach using different *in vitro* test systems and animal models is ideal in planning intervention trials in which advantage is taken of information which may reflect the carcinogenic process in humans. The popularity of rooibos and to a certain extent honeybush herbal teas has increased tremendously the past few years both locally and abroad. In addition to being enjoyable, safe and economical beverages, they provide a natural, rich source of compounds beneficial to human health. Results from this study and others strongly suggest that the consumption of rooibos and honeybush teas may play a role in improving the overall health that could alleviate the cancer burden in humans.

Future directions

Future studies will be aimed at elucidating the possible mechanisms involved by which rooibos and honeybush teas may affect the induction, growth and subsequent progression of specific cancers. The establishment of biomarkers to be used in clinical trials with relation to tea consumption will be important to evaluate the efficacy of human intervention studies.

CHAPTER 9

Public Awareness and Understanding of Science and Technology

(PAUSET)

"We must take science to the people. All of us are fond of quoting Pandit Jawaharlal Nehru's famous words paying a tribute to your community – namely, that 'Scientists are a minority in league with the future'. This is true. But let us also remember that a bright future can be realized only when science is in league with the majority of our society."

Shri Atal Bihari Vajpayee
(Prime Minister of India – 2003)

"Science and Technology (S&T) are essential components of the government's strategy for creating the South Africa of the future. The importance of S&T is recognised outside government as well, by other political parties, by business, the higher education sector, the science councils, labour, NGOs and civil society."

B.S. Ngubane 1996
Minister of Arts, Culture, Science and Technology

1. Extract from White Paper on Science and Technology – Preparing for the 21st Century

"Access to information is empowering, enabling people to monitor policy, lobby, learn, collaborate, campaign and react to proposed legislation. It is also one of the most powerful mechanisms through which social and economic progress can be achieved. The democratization of society and elimination of poverty can only occur if people have equal access to the services and resources they need to perform their productive tasks. Democracy implies being aware of choices and making decisions. The extent to which this is possible depends largely on how much information is available to the people and how accessible it is. For the national system of innovation to become effective and successful all South Africans should participate. This requires a society which understands and values science, engineering and technology and their critical role in ensuring national prosperity and a sustainable environment. This, in turn, requires that S&T information be disseminated as widely as possible in ways that are understood and appreciated by the general public.

Recent history has demonstrated the potential of technology to improve the quality of people's lives. Yet disadvantaged populations in general and women in

particular, especially those in rural areas, have little access to information about these technologies. To date, a combination of factors have prevented them from gaining equitable access to the information they need and have thus limited their ability to participate more fully in the transformation process in South Africa.

A campaign to promote awareness and understanding of S&T and of its importance will have two key elements, namely promoting S&T literacy on the one hand, and promoting the power of S&T on the other. These programmes would include:

- increasing familiarity with the natural world
- promoting understanding of some of the key concepts and principles of S&T
- demonstrating that science, engineering and technology are social tools and
- fostering the ability to use S&T knowledge in ways that enhance personal, social, economic and community development.

The deficiencies of the current system are multifaceted. The solution of this problem requires an innovative approach in itself. All available SET institutions in South Africa should be actively involved in such an initiative.

Government will institute via DACST the delivery of S&T public awareness programmes in collaboration with consortia of institutions, including societies for the advancement of science, professional associations, academies of science, science museums and libraries, media (printed and electronic), educational institutions and private business. (www.polity.org.za/html/ govdocs/white_papers/scitech.html).

2. Contributions of this study towards PAUSET

The reporting of science in South Africa, and for that matter elsewhere in the world as well, poses a challenge. The public and the tea industry should be aware of the implications of current scientific research which may impose directly on their life and quality of life. In order to accomplish this, efforts are made to ensure credible, factual, balanced and relevant information transfer to the public. The current study allowed for many opportunities and occasions to share our knowledge about the possible health benefits of these two endemic herbal teas. Contributions were made in the following areas:

(i) Lay Publications

Two brochures entitled: "Rooibos Tea – A Unique South African Herbal Tea" (Fig. 1) and "Honeybush Tea – A Healthy Beverage from the Cape Fynbos" (Fig. 2) were compiled in conjunction with the Agriculture Research Council. These were specifically designed for the layman. Countless enquiries from the public, herbal tea industry, producers and distributors about the health benefits of these two herbal beverages sparked the initiative. The brochures will continually be updated as the science around rooibos and honeybush teas are elucidated. Distribution of the brochures among healthcare workers, e.g. dieticians, school learners and teachers has proved to be very successful in educations initiatives.

Numerous other lay "publications" in the form of news paper articles and radio and television interviews have been produced and are summarised in Table 1.

(ii) Guest lectures

The following guest lectures were given, mainly as a result from requests from the local herbal tea industry and/or herbal tea distributors:

2000: Health properties of South African herbal teas: Oral presentation at Boise Centre on the Grove, Boise, Idaho, USA, 17 November 2000.

2001: Guest speaker at the opening ceremony of the Rooibos Association of Japan in Tokyo, 18 October 2001.

2002: Presentation at Rooibos Ltd, Clanwilliam, South Africa re scientific research done on rooibos (*Aspalathus linearis*) at PROMEC Unit of the Medical Research Council of South Africa, 13 November 2002.

2003: Workshop presented at the BOSS 2003 (Business Opportunities for Singaporeans) entitled: "Health promoting properties of Rooibos – a Unique South African herbal tea" held at the Singapore Expo Hall, Singapore, 30 August 2003.

Invited guest speaker at event launch of Roibo's rooibos tea held in the Raffles Town Club, Singapore, 2 September 2003.

Invited guest speaker at the press launch of Roibo's rooibos tea held in the Corus Hotel Ballroom, Kuala Lumpur, Malaysia, 4 September 2003.

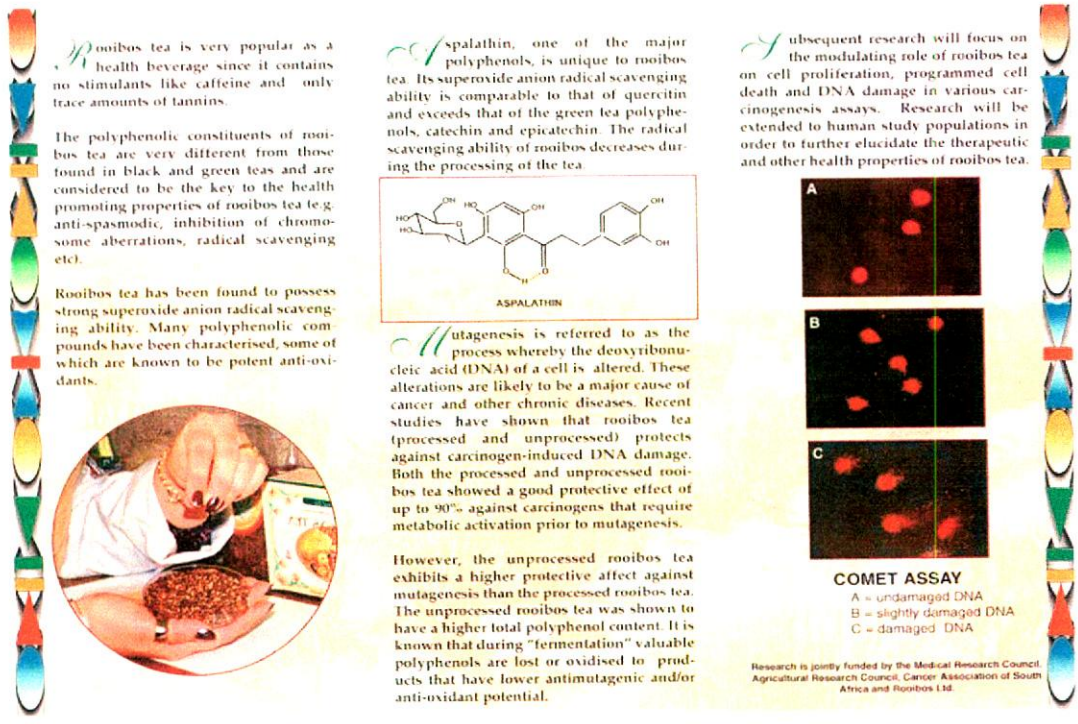

Fig.1. Rooibos tea brochure

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ARC • LNR

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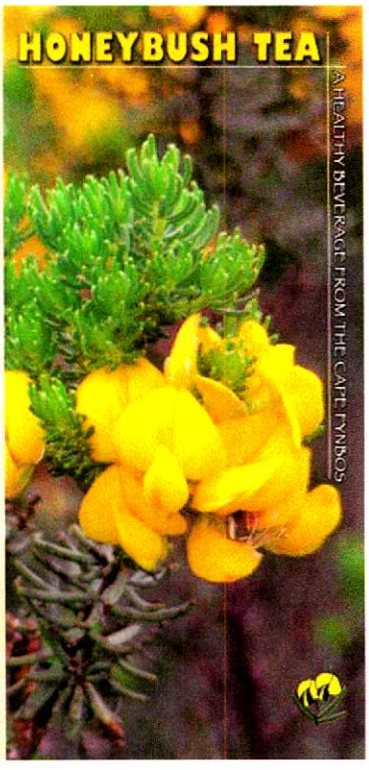
www.mrc.ac.za/promec/contents.htm

MRC

HONEYBUSH TEA

A HEALTHY BEVERAGE FROM THE CAPE FYNBOS



Introduction

Honeybush tea is an indigenous South African herbal beverage, produced from the fynbos plant, *Cyclopia*. Processing is needed to develop the pleasant, sweet, honey-like flavour and brown colour. The absence of caffeine, low tannin content and the presence of antioxidants and antimutagens make this herbal tea a healthy alternative to other warm drinks and beverages. Enhanced antioxidant and antimutagenic potencies are obtained for "green" honeybush, processed in such a manner as to minimise chemical changes.

Traditional use

Use of honeybush tea also known as "bossiestee", spans a few centuries. It is widely believed that the plant has been harvested since the 1700's. As early as 1830 medicinal properties was reported for *C. genistoides*. An infusion or decoction was used as an expectorant for chronic catarrh and pulmonary tuberculosis. Early colonists used it as a restorative.

Anecdotal evidence also suggests that honeybush tea

- cures chronic restlessness,
- alleviates vomiting, stomach cramps and spastic colon,
- aids digestion,
- brings relief to arthritis sufferers, and
- alleviates the symptoms of psoriasis and menopause.

Honeybush as herbal tea

It is unknown at what stage honeybush tea was first processed ("fermented") and became popular as a beverage for everyday use, rather than being used for medicinal purposes. Different *Cyclopia* species were harvested at flowering and processed during the earlier part of the previous century. The tea was prepared for home consumption and enjoyed as part of the normal diet. Today *C. intermedia* ("bergtee") and *C. subternata* ("vleitee") are of importance in terms of quantities produced, while the lesser-known *C. genistoides* ("kustee") is gaining in prominence as more and more plantations are established.

Substantiation of health benefits

The benefits of regular consumption of honeybush tea are only now in the process of being unraveled. Investigations on the antioxidant and antimutagenic/anticarcinogenic properties of honeybush tea are in progress at ARC Infruitec-Nietvoorbij and MRC (PROMEC Unit), but in future other potential medicinal properties will receive attention to fully exploit honeybush tea as a health beverage.

Antioxidants have the ability to scavenge free radicals therefore could potentially protect cells in the human body against oxidative damage. An imbalance in the cell's protective mechanisms against oxidative damage is believed to be a contributing factor in a broad spectrum of diseases including atherosclerosis, inflammatory diseases such as arthritis, heart diseases, Alzheimer's disease and cancers.

In vitro antioxidant activity, i.e. the ability to scavenge the superoxide anion radical and protect cell membranes against oxidation, has been demonstrated for infusions prepared from different honeybush species. Several of the phenolic compounds present in *C. intermedia* exhibit antioxidant activity, but potency varies, depending on their chemical structure and the test system used. An animal study has shown that honeybush increased the antioxidant capacity of the liver.

Antimutagenicity is the ability to prevent gene mutations which could lead to cancer development. Results from an experimental animal study suggest that honeybush tea could protect against cancer induction by altering the conversion of carcinogens compounds in the body making them less toxic.

Although some data are available on the absorption of phenolic compounds that are also present in honeybush, it not known to what extent the bio-active constituents are absorbed or metabolically transformed to exhibit their biological properties. Their absorption from the human gut is a key aspect that needs to be investigated to fully understand the role and health properties of these compounds.

Fig.2 Honeybush tea brochure

Table 1 Lay Publications

Media	Sources	Title
Printed media	MRC News, Vol 30, no 4, November 1999	Anyone for tea? – Izelle Theunissen (News Editor)
	Sunday Times, 20 February 2000	SA teas keep cancer at bay – Laurice Tait (Health reporter)
	Fair Lady, 19 January 2000	Down to a tea – Karene d Plessis (Health Line reporter)
	Die Burger, 24 August 2000	Heningbostee se onskatbare potensiaal nog grootliks onontgin – Laetitia Watson (reporter)
	South African Honeybush Tea Association, Newsletter July 2003, no 8.	Honeubsh tea and cancer prevention – E. Joubert, JL Marnewick (scientists)
	Information leaflet included in press kit for launches of Roibo's rooibos in Singapore and Malaysia September 2003.	Health promoting Properties of Rooibos – a Unique South African Hebal Tea" - JL Marnewick (scientist).
	HerbalGram, USA, entitled: (www.herbalgram.org/herbalgram)	Rooibos: This herbal tea serves a cupful of antioxidants without caffeine or tannin – Laurie Erickson (health reporter)
Television	Interview by Daniel Lee, of the Seoul Broadcasting System (South Korea), 16 May. The interview forms part of a documentary film promoting South Africa as a tourist destination. Rooibos tea was included as it only grows in South Africa and is popular in Korea. The film crew spent more than 3 hours filming activities in the PROMEC Unit.	

2004: Invited guest speaker at Royal Health Service's National Royal Health Workshop for dietitians on the Health promoting properties of Rooibos tea. The workshop was held at Panorama Medi Clinic, 20 January 2004.

(iii) Empowerment of school teachers and learners

A workshop was held at the MRC (7 March 2003) to accommodate twenty five science teachers from schools in the Western Cape area. One of the sessions addressed the question of "How a scientific investigation should be conducted", using the Rooibos project as an example.

The MRC's Events Management Community Liaison Office extended an invitation to address the grade 10 to 12 learners from Bonteheuwel High School (17 March 2003) as well as Bernadino Heights High School, Kraaifontein (29 July 2003) on the health promoting properties of rooibos tea.

A presentation on the "Health promoting properties of Rooibos and honeybush teas" was given to 55 B Sc students at 2nd or 3rd year level from the Science Faculty of The University of Zululand, 23 September 2003.

(iv) Capacity development

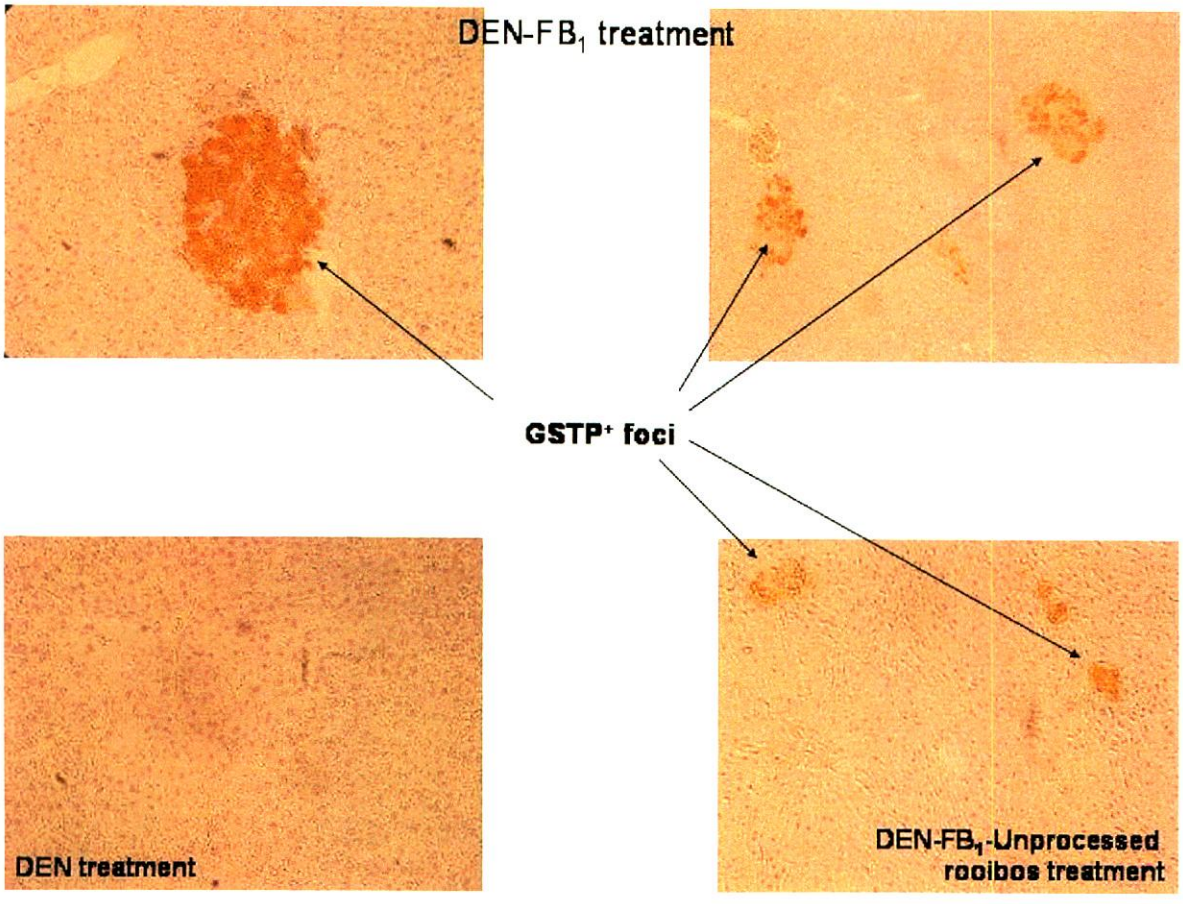
Since January 2000 to date, 15 students from both local and abroad universities and colleges have been trained in the AMES assay, isolation of primary hepatocytes, tissue culture techniques and execution of animals studies. These students were mostly post-graduate students requiring practical experience for completion of their degree/ qualification.

In conclusion, when conducting a scientific investigation, sound basic research is required but it is of utmost importance that the research findings have to be effectively communicated not only to the scientific community, but also to the public. This aspect was recently emphasized by Kenneth Olden, Director of the NIEHS, that the research process should be a "two-way street – communication does not simply flow from the ivory towers, but that people charged with setting research agendas should be responsive to the needs and messages of the public they serve".

"Think like a wise man but communicate in the language of the people"
William Butler Yeats (1865-1939)

Addendum A:

Liver sections of rats treated with DEN-FB_1, DEN and DEN-FB_1-unprocessed rooibos tea, (Chapter 6) stained to detect GSTP altered hepatocytes.



Addendum B:

Visual aspects of the two stage mouse skin carcinogenesis model performed in this study (Chapter 7).